Occurrence and Epidemiology of *Fusarium* Species in Barley and Oats

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich, 2017

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Summary

Fusarium head blight (FHB) is one of the most devastating cereal diseases, caused by a complex of different *Fusarium* species. For wheat, *F. graminearum* (FG) belongs to the most frequently occurring species worldwide and in Switzerland. The infection results not only in yield reductions, but also in mycotoxin contamination of harvested grains, threatening human and animal health. The cereal species have an impact on the occurrence of the most dominant *Fusarium* species and their respective mycotoxins. Furthermore, cropping factors influence the fungal inoculum and infection conditions in the field. Moreover, the characterisation of weather factors is important to better understand the biology of a given fungal species and to ultimately develop forecasting systems, which help to predict a potential contamination with mycotoxins in cereal based food and feed. The first objective of the present study was to identify the most occurring *Fusarium* species and mycotoxins in Swiss barley and oats and to discover potential influencing cropping factors. The second objective was to investigate under controlled conditions the effect of temperature and humidity conditions on infection by the dominant *Fusarium* species in barley and oats as well as to examine the effect of weather conditions on spore deposition, infection and toxin accumulation in the field.

To achieve the first objective, a Swiss wide *Fusarium* monitoring for barley and oats was conducted over two and three years, respectively. In barley, FG was the dominant *Fusarium* species and deoxynivalenol (DON) the main mycotoxin. In contrast, *F. poae* (FP) and T-2/HT-2 were the most prominent *Fusarium* species and mycotoxins in oats. Moreover, a large dataset with the most important agronomic factors that could influence a *Fusarium* infection was established. The analysis of these factors revealed that the previous crop maize in combination with reduced or no tillage leads to an increased infection with FG and elevated DON contents in barley. Nevertheless, additional chopping of the maize crop residues reduced infection and contamination. The cropping system (organic, extensor, or PEP) did not show an effect on the disease development. Hence, instead of a single agronomic measure, the entire cropping system should be taken into account to retrieve factors influencing the respective *Fusarium* species. For oats, small grain cereals as previous crops enhanced the contamination with T-2/HT-2, mainly produced by *F. langsethiae* (FL), and autumn sown compared with spring sown oats, resulted in higher incidences of both FP and FL. Hence, the cultivation of maize before barley and of small grain cereals before oats, in particular in reduced tillage systems, should be avoided.

For the second objective, the impact of various combinations of different temperatures (10, 15, 20 °C) and 99% relative humidity (rH) durations (4, 8, 12 h) on infection by FG in barley and by FP or FL in oats was examined. Additionally, the most favourable growth stage for FL inoculation in oats was elucidated. Irrespective of the humidity duration, the highest FG infection and DON contamination in barley was observed at inoculation temperatures at 15 °C, whereas for oats, 10 °C were shown to enhance the infection with FP or FL and the nivalenol or T-2/HT-2 contents, respectively. With respect to growth stage, the beginning and mid of anthesis revealed to be the most favourable for FL infection and T-2/HT-2 accumulation, which excludes a significant infection before anthesis. Due to the insufficient data on collected FP and FL spores, the interpretation of spore deposition in the field as well as infection and toxin concentrations represents a challenge. Nevertheless, it is unknown whether FP and FL produce ascospores and whether these or conidia are transported by rain and/or wind, hence, thorough studies on the spore dispersal should be conducted. Subsequently, the obtained data in relation to weather variables could then be used to predict the spore dispersal. Overall, this study contributed to reveal the *Fusarium* species spectrum in barley and oats as well as to identify the most important cropping factors to avoid the infection risk. It also provided a first insight into the epidemiology of FG in barley and FP/FL in oats. The obtained results are of crucial importance to ultimately develop cropping strategies and forecasting systems to ensure the safety of barley and oats for food and feed.

Zusammenfassung

Die Ährenfusariose ist eine der gefährlichsten Getreidekrankheiten, welche durch verschiedene *Fusarium*-Arten verursacht wird. Bei Weizen ist *F. graminearum* (FG) die am häufigsten vorkommende Art weltweit und in der Schweiz. Eine Infektion führt nicht nur zu Ernteverlusten, sondern auch zur Belastung des Ernteguts mit Mykotoxinen, welche die Gesundheit von Menschen und Tieren gefährden können. Zwischen den Getreidearten gibt es Unterschiede bezüglich der jeweils vorherrschenden Fusarienarten und den dominantesten Mykotoxinen. Weiterhin beeinflussen Anbaufaktoren wie Vorfrucht und Bodenbearbeitung das Pilzinokulum und die Infektionsbedingungen im Feld. Zusätzlich sind Erkenntnisse über beeinflussende klimatische Faktoren erforderlich, um sowohl die Biologie der jeweiligen Pilzart besser zu verstehen als auch um Prognosesysteme zu entwickeln, wodurch eine potentielle Kontamination von Getreide erkannt werden kann. Das erste Ziel dieser Arbeit war die Identifikation der dominantesten Fusarien-Arten und –Mykotoxine in Schweizer Gerste und Hafer, sowie die Untersuchung von befallsbeeinflussenden Anbaufaktoren. Das zweite Ziel war, unter kontrollierten Bedingungen den Einfluss von Temperatur und Feuchtigkeit auf die Infektion mit den dominantesten Fusarien in Gerste und Hafer zu untersuchen sowie unter Feldbedingungen den Einfluss klimatischer Faktoren auf den Sporenflug, die Infektion und Toxinbelastung zu erforschen.

Um diese Ziele zu erreichen, wurde zunächst ein schweizweites Monitoring über zwei Jahre für Gerste bzw. drei Jahre für Hafer durchgeführt. Bei Gerste waren FG und Deoxynivalenol (DON) die bedeutendste Art bzw. das Haupttoxin, während bei Hafer *F. poae* (FP) und T-2/HT-2 die vorherrschende Art bzw. die häufigsten Mykotoxine waren. Die Analyse der Anbaufaktoren zeigte, dass bei Gerste die Vorfrucht Mais in Kombination mit reduzierter Bodenbearbeitung oder Direktsaat die Infektion mit FG und die Belastung mit DON förderte, wobei dieser Effekt durch eine zusätzliche Zerkleinerung der Mais-Erntereste minimiert wurde. Das Anbausystem (biologisch, integriert oder ÖLN) hatte keinen Einfluss auf die Krankheitsentwicklung. Statt die Wirkung einzelner Faktoren zu beobachten, sollte daher stets das gesamte Anbausystem betrachtet werden. Bei Hafer erhöhten kleinkörnige Getreide als Vorfrucht die Kontamination mit T-2/HT-2, welche hauptsächlich von *F. langsethiae* (FL) gebildet werden, und Herbst- im Vergleich zu Sommersaaten führten zu einem höherem Vorkommen mit FP und FL. Daher sollten, insbesondere bei nichtwendender Bodenbearbeitung, die Vorfrüchte Mais bei Gerste und kleinkörnige Getreide, bei Hafer vermieden werden. Zur Erreichung des zweiten Ziels wurde die Wirkung verschiedener

Kombinationen aus Temperatur (10, 15, 20 °C) und Dauer (4, 8, 12 h) der 99% relativen Luftfeuchte (rF) auf die Infektion mit FG in Gerste, sowie auf diejenige von FP und FL in Hafer untersucht. Zudem wurde geprüft, welche Wachstumsstadien von Hafer zu der stärksten Infektion mit FL führen. Unabhängig von der Feuchtigkeitsdauer wurden bei Gerste die höchste FG- Infektion und DON-Belastung nach einer Inkubation bei 15 °C erzielt, wohingegen bei Hafer 10 °C-Inkubationen in den höchsten FP- und FL-Infektion sowie in höheren Nivalenol- und T-2/HT-2-Werten resultierten. Die Infektionen zu Beginn und Mitte der Blüte führten zu den höchsten FL-Infektionen. Die geringe Datenerfassung zum Sporenflug in den Feldexperimenten ermöglicht kein abschliessendes Fazit. Da die Produktion von Askosporen bei FL und FP unbekannt ist und ob diese oder Konidien durch Wind bzw. Regen verbreitet werden, sollten weitere Experimente zum Sporenflug durchgeführt werden. Diese Daten können dann genutzt werden, um in Verbindung mit Wetterparametern den Sporenflug zu prognostizieren. Diese Arbeit erlaubte es, das *Fusarium*-Artenspektrum in Gerste und Hafer zu erfassen und die wichtigsten befallsbeeinflussenden Anbaufaktoren zu identifizieren, welche das Infektionsrisiko reduzieren können. Zudem wurde ein erster Einblick in die Epidemiologie von FG in Gerste sowie von FP/FL in Hafer erhalten. Die erzielten Erkenntnisse sind von entscheidender Bedeutung, um schlussendlich Anbausysteme und Prognosesysteme zu entwickeln, welche die Sicherheit von Gerste und Hafer als Lebens- und Futtermittel gewährleisten.

1 General introduction

The production of crops is connected with the challenge to withstand abiotic and biotic influences which reduce plant yield (Oerke 2006; Solomon 2011). About 10% of the global food production is lost due to plant diseases (cited in Strange and Scott 2005). Fusarium head blight (FHB) is one of the most devastating fungal disease in cereals. It is caused by phytopathogenic fungi from the genus *Fusarium* which belongs to the group of Ascomycota (Parry et al. 1995; Gilbert and Haber 2013). This genus was first described by H.F. Link in 1809 as *Fusisporium* (cited in Summerell and Leslie 2011).

An outbreak of FHB substantially reduces yield, mainly by the reduction of the thousand grain weight due to inhibition of the nutrient supply to upper spikelets. The yield losses can range between 2 to 40% under natural conditions (Parry et al. 1995). A complete economic loss can occur, since harvested goods containing mycotoxins, which in consequence do not meet quality and food safety standards, cannot be sold (Nganje et al. 2004).

The expression mycotoxin was first used after a veterinary crisis in England in 1961. Due to a contamination of peanut meal with aflatoxins caused by the filamentous fungus *Aspergillus flavus*, thousands of animals died (reviewed in Richard 2007). Today, more than 400 mycotoxins are known and grouped according to their toxic activity as mutagenic, carcinogenic or teratogenic (causing developmental malformations) (Bennett and Klich 2003). Five fungal genera are mainly known to produce mycotoxins: *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium* and *Penicillium.*

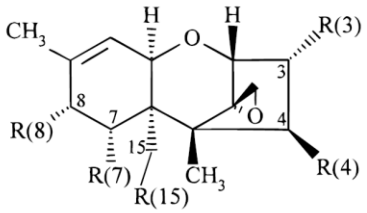
If cereals contaminated with these mycotoxins are used for human consumption or animal feed, they can cause health difficulties such as disruption of the gastrointestinal tract, anaemia, leukopenia and skin irritation (Milićević et al. 2010; Whitlow et al. 2010). Furthermore, trichothecenes can inhibit the mitochondrial function and protein synthesis as shown *in vitro* and *in vivo* (Oldham et al. 1980; Rosenstein and Lafarge-Frayssinet 1983; Ngampongsa et al. 2013).

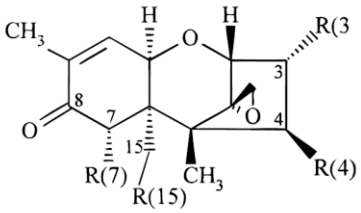
Mycotoxins produced by *Fusarium* species belong to the family of trichothecenes, a group of 150 structurally similar compounds, whose common feature is the 12,13-epoxytrichothecene skeleton and an olefinic bond with various side chain substitutions (Dänicke et al. 2006). Among the trichothecenes, four types can be distinguished by their different chemical structure at the C-8 position (Figure 1): type A includes T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol, type B contains deoxynivalenol and its acetylated forms, nivalenol and

fusarenon-x, whereas type C and type D are not produced by *Fusarium* species (Ueno 1985; Dänicke et al. 2006; Bennett and Klich 2003). Generally, the type A trichothecenes are known to be more toxic than type B (Rotter et al. 1996; Desjardins 2006). Compared with DON, T-2 and HT-2 are ten times more toxic (Ueno et al. 1973). But also within one trichothecene type, the toxicities differ. In mice, the NIV toxicity was shown to be higher than that of DON (cited in Kongkapan et al. 2016) (Cheat et al. 2015).

The *Fusarium* species spectrum varies between the different cereal types and between the different geographic areas and thus the mycotoxin contamination is varying as well because different *Fusarium* species produce different mycotoxins (Osborne and Stein 2007; Vanheule et al. 2014; Desjardins 2006). Moreover, the occurrence of mycotoxins is depending on various variables such as weather conditions, host species, cropping factors and storage conditions (Placinta et al. 1999). Furthermore, co-occurrence of several *Fusarium* species within one field has been observed. Thus, it is expected that humans and animals are exposed to a mixture of mycotoxins (Vanheule et al. 2014; Xu et al. 2008; Milićević et al. 2010). Consequently, the European Union has set in 2006 maximum limits for a number of *Fusarium* toxins in food and guidance values in animal feed (The European Commission 2006) which were adopted in the Swiss legislation (Federal Department of Home Affairs 2016).

To minimise the risk of a *Fusarium* infection and thus the contamination of cereals with mycotoxins, several control measures including cropping techniques, fungicide application or biological control agents are available (Blandino et al. 2012; Parry et al. 1995; Nguyen et al. 2017). Furthermore, knowledge on the epidemiology of *Fusarium* species, including the most susceptible growth stages, is the most important point to understand the disease and to develop efficient control measures.





Type A Trichothecenes							Type B Trichothecenes					
	MW	**R(3)**	**R(4)**	**R(7)**	**R(8)**	**R(15**		**MW**	**R(3)**	**R(4)**	**R(7)**	**R(15)**
NEO	382.2	OH	OAc	H	OH	OAc	**NIV**	312.1	OH	OH	OH	OH
DAS	366.2	OH	OAc	H	H	OAc	**DON**	296.1	OH	H	OH	OH
HT-2	424.2	OH	OH	H	i-Val	OAc	**F-X**	354.1	OH	OAc	OH	OH
T-2	466.2	OH	OAc	H	i-Val	OAc	**3-ADON**	338.1	OAc	H	OH	OH
VOL	266.2	H	OH	H	H	OH	**15-ADON**	338.1	OH	H	OH	OAc

MW: Molecular weight
OAc: acetyl
i-Val: *iso*-valeryl

Figure 1: Structures of type A- and B-trichothecens; NEO: neosolaniol, DAS: diacetoxyscirpenol, HT-2: HT-2 toxin, T-2: T-2 toxin, VOL: verrucarol, NIV: nivalenol, DON: deoxynivalenol, F-X: fusarenon-X, 3-ADON: 3-acetyldeoxynivalenol, 15-ADON: 15-acetyldeoxynivalenol (Berger et al. 1999).

1.1 *Fusarium* species taxonomy

Today, more than 300 *Fusarium* species have been identified and are grouped into 17 different species sections (Aoki et al. 2014; Munkvold 2017; O'Donnell et al. 2013). In the past, the *Fusarium* taxonomy was based only on morphological identification. Biological and phylogenetic species concepts were introduced during the late twentieth century. Today, molecular methods are used to identify the species (Aoki et al. 2014; Munkvold 2017). Since 2013, the dual nomenclature was abandoned and only the anamorph names are used today (Hawksworth 2012). But for the sake of completeness, the teleomorph names will be mentioned as well. The main *Fusarium* species in barley and wheat (*F. graminearum*) (Parry et al. 1995), as well as the main species in oats (*F. langsethiae* and *F. poae*) (Bernhoft et al. 2012; Yli-Mattila et al. 2008) belong to the *sambucinum* species complex (Figure 2).

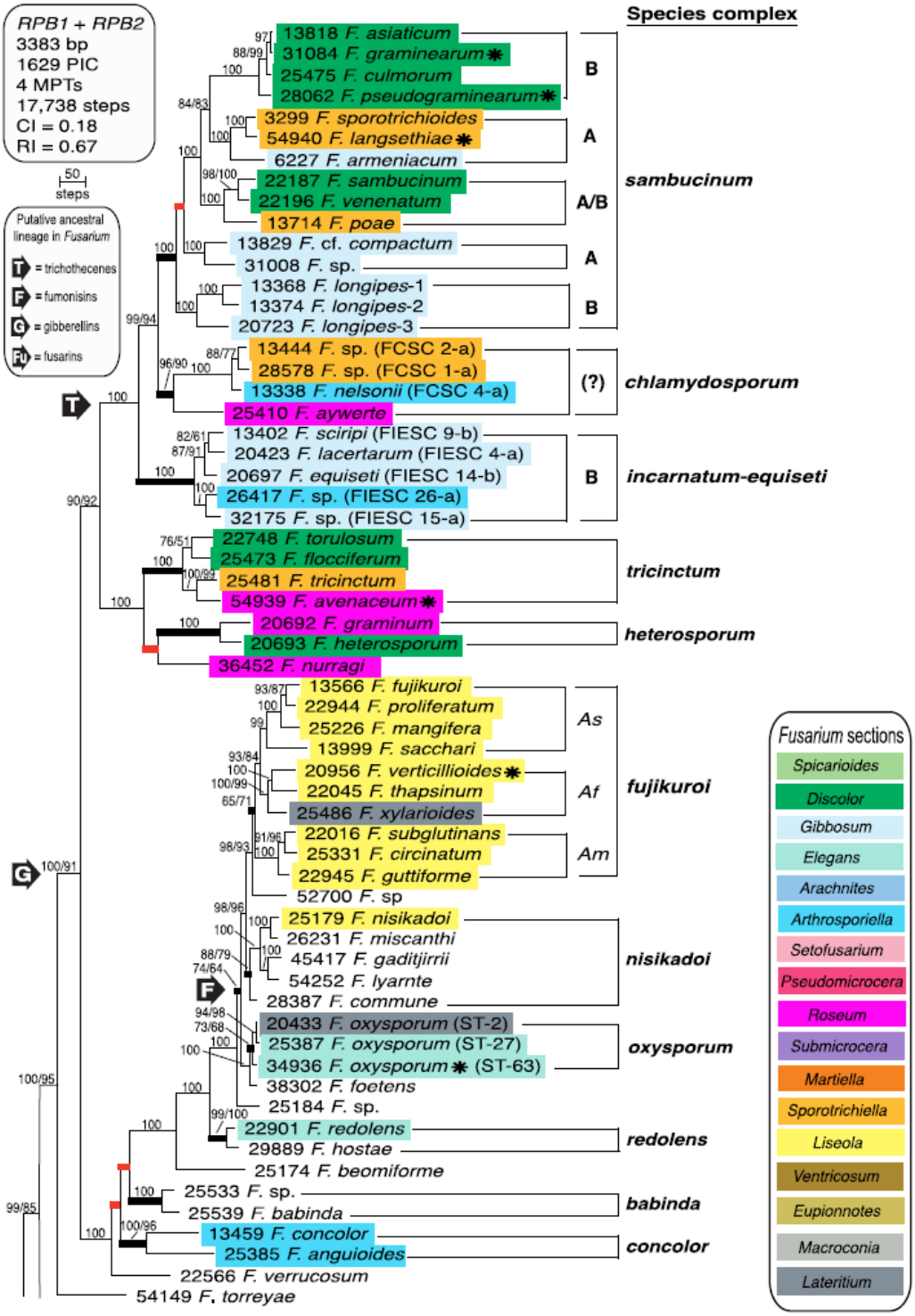


Figure 2: Extract of the parsimonious tree, showing the main *Fusarium* sections and species complex within the sambucinum complex; A and/or B type trichothecene production is mapped onto sambucinum subclades species complex. Numbers above internodes represent maximum likelihood (ML) and maximum parsimony (MP) bootstrap support. Thickened blacked branches identify six basal non-*Fusarium* lineages; thickened red branches identify nine internodes that were not supported by ML and MP bootstrapping. Bold black asterisks identify seven species whose whole genome sequences were surveyed for secondary metabolite gene clusters. Within the fujikuroi species complex, three biogeographically structured subclades are identified: As = Asian, Af = African, Am = American. (O'Donnell et al. 2013).

1.2 Biology and epidemiology of different *Fusarium* species

1.2.1 *Fusarium graminearum*

The worldwide most occurring *Fusarium* species in cereals is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* Schwein, (Petch) (Parry et al. 1995). *Fusarium graminearum* (FG) is a facultative parasitic fungus with only a short pathogenic phase on the living plant in the FHB disease cycle (Sutton 1982; Parry et al. 1995; Osborne and Stein 2007). *F. graminearum* produces relatively slender, sickle-shaped macroconidia with a distinctly foot-shaped basal cell (Leslie and Summerell 2006). Under culture conditions on potato dextrose agar (PDA), the fungus grows rapidly and produces a dense aerial mycelium, white to yellow in colour and the cultures form red pigments in agar media (Leslie and Summerell 2006).

The fungus is able to overwinter saprophytically as mycelium on crop residues of different plants such as maize, wheat or canola (Osborne and Stein 2007; Sutton 1982; Fernandez 2007; McMullen et al. 2012). The presence of inoculum can last up to two years, as shown by Pereyra et al. (2004). For long-term survival, thick-walled chlamydospores can be produced, which can survive in the soil for several years until adequate infection conditions arise (Parry et al. 1995). In spring, when temperatures rise and the humidity is sufficiently high, FG enters its sexual stage and starts to produce perithecia, fruiting bodies, on the surface of the crop debris (Figure 3). Inside these perithecia, the ascospores are produced in tubular asci (eight ascospores per ascus) (Trail and Common 2000). The optimal conditions for the production of perithecia and ascospores are 24 °C to 29 °C and 25 °C to 28 °C, respectively (Sutton 1982; Doohan et al. 2003; Dufault et al. 2006). The ejection of ascospores is favoured by high humidity and moderate temperatures, with an optimum near 16 °C but in a range between 10 °C and 30 °C (Doohan et al. 2003; Sutton 1982; Paulitz 1996). The mature perithecia discharge their ascospores via turgor pressure and can reach a calculated speed of 35 m s^{-1} and an acceleration of 8.5 x 10^6 m s^{-2}, which is the fastest acceleration ever recorded in a biological system (Trail et al. 2005; Trail 2007). These ejected ascospores are the primary inoculum and can be dispersed by wind and rain-splash over kilometres and meters, respectively (Dill-Macky and Jones 2000; Osborne and Stein 2007; Paul et al. 2004).

Once these ascospores reach the flowering cereal ear, which is the most susceptible growth stage for an infection, it is able to infect the plant (Parry et al. 1995; McMullen et al. 1997). On the anthers, it penetrates with its germ tube through the stomata of the glumes and spreads

subcuticularly along the stomata. Subsequently, the fungus attacks the parenchyma cells of the glumes and further sporulation happens 48 to 76 h after infection. The asexual spores, the conidia, are now formed in short asexual cycles and allow a spread into other parts of the wheat head (Pritsch et al. 2000; McMullen et al. 1997).

The conidia production is the mass reproduction of the fungus throughout the ongoing growing season and is favoured by moist and warm weather (Parry et al. 1995; Fernando et al. 1997).

FHB symptoms begin to develop as small, brownish spots at the glume or on the rachis. As the spores proliferate, they become salmon pink and eventually discoloured. At the beginning of the milk stage (DC 75) (Zadoks et al. 1974), single spikelets or whole sections of the ear are chlorotic and obtain a bleached appearance. The nutrient and water supply to the upper ear is cut off which reduces the kernel development. The harvested grains are frequently shrivelled and of brown, grey or pinkish colour (Parry et al. 1995). If infected crop residues remain in the field, they can form the inoculum source for the following growing period.

Shortly after infection, FG is able to produce different mycotoxins (Miller 2008; Evans et al. 2000), including type B trichothecenes (deoxynivalenol [DON], 3- and 15-acetyl DON, nivalenol [NIV]) and zearalenone (Pasquali et al. 2016; Osborne and Stein 2007; Desjardins 2006). The amount of DON production can vary greatly depending on the environmental conditions. High moisture after anthesis and temperatures above 20 °C during the infection period were shown to enhance the DON production (Xu et al. 2007a). Depending on the storage conditions (insufficient drying of grains, high temperatures), the mycotoxin content in the grains can still increase after harvest (Birzele et al. 2000).

In humans and animals, DON is known to affect the digestive system and major organ functions, which can result in vomiting or diarrhoea (reviewed in Rotter et al. 1996). Also the susceptibility to bacterial, fungal and viral diseases is increased (Bondy and Pestka 2000) and it affects the human lymphocyte cytokine production and thus can lead to apoptosis (Shifrin and Anderson 1999; Sun et al. 2002). Due to its cytotoxicity, the European Commission has issued a maximum limit of 1250 µg kg^{-1} in unprocessed cereals intended for human consumption (The European Commission 2006).

Particularly in pigs, DON can lead to feed refusal and vomiting, which resulted in the common name “vomitoxin”. Also a reduced productivity of livestock was observed upon feeding DON contaminated grain (Bennett and Klich 2003; Wegulo 2012).

Another unwanted implication is the influence on baking and brewing quality. The processing of DON contaminated grains can result in decreased loaf volume and in a stickier and difficult to mould dough (Dexter et al. 1996). In breweries an increased gushing potential and reduced

lautering performance was reported (Sarlin et al. 2005; Wegulo 2012). Since DON and most mycotoxins are relatively heat stable, they can persist during various processing methods and thus remain in the final product (Milićević et al. 2010; Vidal et al. 2016; Vidal et al. 2014; Neme and Mohammed 2017).

Zearalenone (ZEA), a β-resorcylic lactone, does not possess an acute toxicity but acts like a nonsteroidal oestrogen, leading to the term mycoestrogen. It competes with the endogenous oestrogen for binding sites on the cytosolic oestrogen receptors, resulting in adverse effects on the structure and function of the reproductive organs (Dänicke et al. 2006; Bennett and Klich 2003; Kuiper-Goodman et al. 1987).

The most sensitive animals to ZEA are female pigs and frequently, unnatural growth of the uterus and mammary enlargement are observed (Diekman and Green 1992). Furthermore, malformation of piglets or even aborts were reported after feeding *Fusarium* infected fodder to pregnant gilts (Sharma et al. 1974).

F. graminearum was studied mainly in wheat and maize, but comparatively less work was done on barley and oats (Elen et al. 2003; Langseth and Elen 1996, 1997; Campbell et al. 2002). As of today, barley is mainly used as fodder, hence with respect to feed safety, it is essential to investigate whether FG occurs frequently in Swiss barley and which are the mainly produced mycotoxins. In addition, the smaller amount of barley used for human consumption needs to be free of mycotoxins to ensure food safety and qualitative food production. The factors that have an impact on infection and contamination have to be elucidated in order to establish cultivation recommendations to reduce the risk.

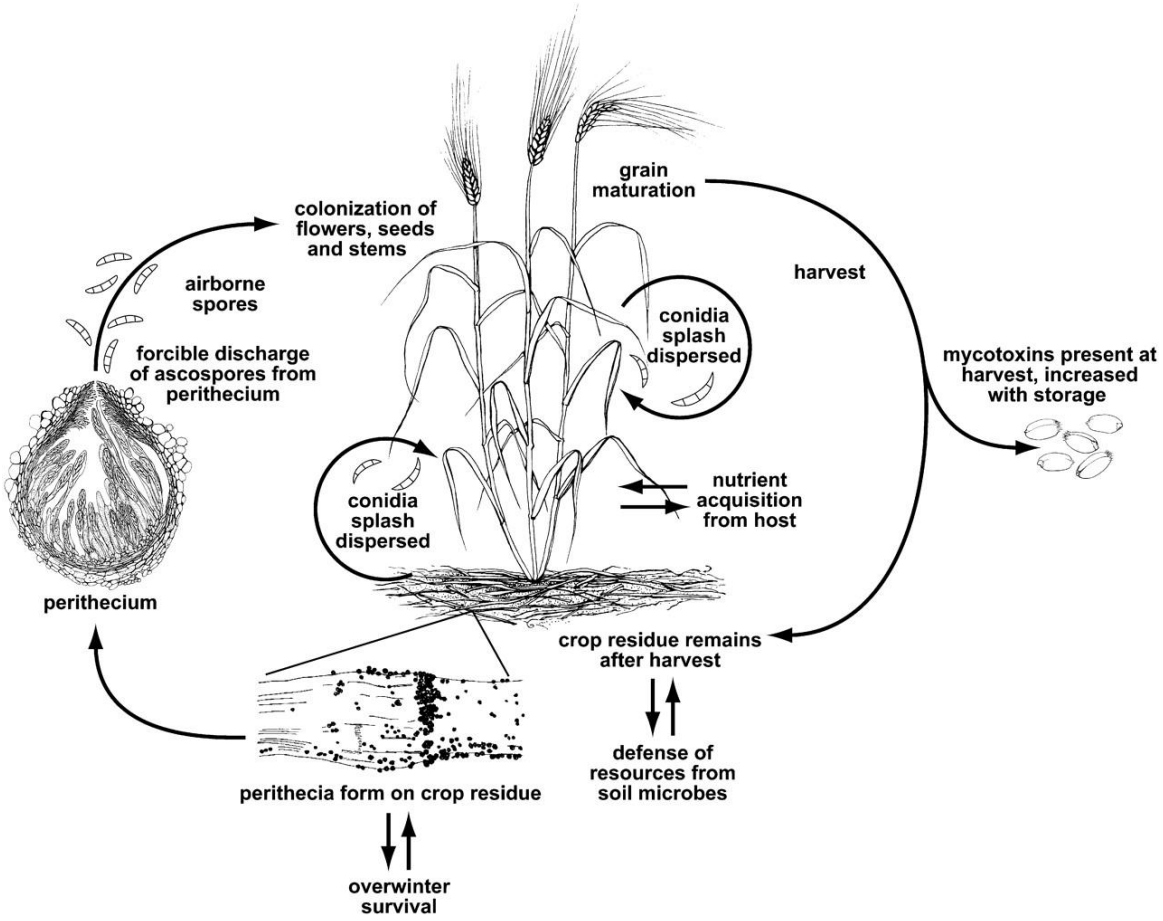


Figure 3: Life cycle of *Fusarium graminearum* (Trail 2009).

1.2.2 *Fusarium poae*

Fusarium poae (FP) was first described as *Sporotrichum poae* by C. H. Peck in 1902 and transferred to the genus *Fusarium* by H. W. Wollenweber in 1913 (cited in Stenglein 2009). *F. poae* typically develops clusters of short branched and unbranched monophialides, microconidia are abundant and global or oval to piriform. Macroconidia are typically sickle-shaped with a foot-shaped basal cell but occur rarely (Leslie and Summerell 2006). On PDA, the fungus produces a dense aerial mycelium, white to pink in colour and produces volatiles with a characteristic fruity odour (Leslie and Summerell 2006). Two mating types (MAT-1 and MAT-2) of FP occur and are transcribed, however, the teleomorph is not known and a cryptic sexual cycle is assumed (Kerényi et al. 2004).

F. poae occurs in different parts of the world including Canada (Bourdages et al. 2006) and several countries in Asia (Sugiura et al. 1993) and in Europe (Yli-Mattila et al. 2008; Ioos et al. 2004; Bottalico and Perrone 2002), with varying incidence depending on the region and the prevailing weather conditions in the respective year (cited in Stenglein 2009).

Parry et al. (1995) reported a wetness period of at least 24 h and temperatures above 15 °C for an optimum infection. The same trend was observed by Xu et al. (2007a), showing a higher incidence of FP and NIV content with increasing wetness duration (36 h) and temperature (25 °C). However, monitoring studies in wheat showed a higher occurrence of FP under dry and warm conditions, probably because these conditions are less favourable for FG development (Xu et al. 2008; Chrpová et al. 2016).

The epidemiology of FP was not studied as detailed as that of FG, thus only limited data is available. It is assumed, that FP colonises the ears of wheat and barley prior to emergence from the boot stage but further penetration processes are unknown (Sturz and Johnston 1983). The splash dispersal was studied indoor with the aid of artificially produced leaves by Hörberg (2002), who showed a transfer of colonies up to 58cm in vertical and up to 70cm in horizontal direction. She concluded that spores on the underside of the leaf can infect the stem base, whilst spores on the upper side could spread further, thus leading to infection of the ear. However, the dispersal of microconidia as well as the role of wind dispersal remains unclear and studies under field conditions are needed.

The role of insects as vectors for FP was reviewed by Parry et al. (1995). It was summarised that insects such as mites and thrips might play an important role in the transportation of FG, *F. avenaceum* and *F. culmorum* inoculum. Still, verification of active transport is needed. The role of alternative hosts is a factor that should be studied in detail, since FP was isolated from various species apart from cereals, such as grasses, tomato and gramineous weeds (Nedělník et al. 2015; Stenglein et al. 2009; Landschoot et al. 2011).

In contrast to FG, infection with FP does not result in the typical FHB symptoms (Figure 4), instead, small necrotic lesions can be observed in wheat and even remains symptomless in oats which enhances the risk of healthy looking grains, contaminated with mycotoxins (Vogelgsang et al. 2008b; Stenglein 2009; Torp and Adler 2004).

Compared with FG, FP is a rather weak pathogen in terms of infection but might have a greater saprophytic ability compared to other *Fusarium* species (Pereyra et al. 2004). Still, depending on the populations, it can produce both, type A and B trichothecenes as well as enniatins, beauvericin and moniliformin (Dinolfo and Stenglein 2014; Thrane et al. 2004). Apart from being a major NIV producer, it can also produce neosolaniol, diacetoxyscirpenol or fusarenone-x (Desjardins 2006). Recently, the occurrence of NIV together with DON was shown to result in synergistic effects in terms of cell toxicity and thus should be monitored further (Yang et al. 2017).

Toxic effects of NIV include the inhibition of protein and DNA synthesis as well as apoptosis (Ryu et al. 1987; Poapolathep et al. 2002). Still, no maximum limits for NIV were set by the EU but the European Food Safety Authority (EFSA) has issued a tolerable daily intake (TDI) of 1.2 µg/kg bodyweight which is slightly higher compared with the TDI of DON (1 µg/kg bodyweight) (EFSA 2013b, 2013a).

Since the knowledge about the epidemiology of FP, such as overwintering survival or sexual recombination is sparse, more detailed research is necessary. Furthermore, the occurrence, toxin producing abilities and influencing cropping parameters in both barley and oats in Switzerland are unknown.

Figure 4: Symptoms of *Fusarium poae* on wheat (left) and of *F. graminearum* on barley (right) (picture: T. Schöneberg, Agroscope).

1.2.3 *Fusarium langsethiae*

Fusarium langsethiae Torp & Nirenberg (FL), formerly known as "powdery *Fusarium poae*", was described as a new species in 2004 (Torp and Nirenberg 2004) in the section *Sporotrichiella*. The former classification was based on its morphological similarity to FP, whereas the mycotoxin production is similar to that of *F. sporotrichioides* Sherbakoff. It differs from FP by its slower growth, less aerial mycelia and the absence of the fruit-like odour on synthetic media (Torp and Nirenberg 2004). The spore morphology is close to that of FP, with

small napiform to globose conidia (Torp and Langseth 1999). Optimum growth conditions were reported to be in the range between 20 to 30 °C (Torp and Nirenberg 2004). Optimal conditions for T-2/HT-2 production in stored oats were reported to be at 25 °C and a water activity (a_w) of 0.97 (Mylona and Magan 2011) whereas Kokkonen et al. (2010) showed a higher production under 15 °C and 0.99 a_w.

It was detected in infected grains of barley (Linkmeyer et al. 2016; Nielsen et al. 2014) and wheat (Dedeurwaerder et al. 2014; Infantino et al. 2014) but is regularly found in oats (Edwards et al. 2012; Bernhoft et al. 2012; Hofgaard et al. 2016a). So far, the detection of FL is mostly limited to Europe but it was also found in western Siberia (Yli-Mattila et al. 2015). The lower detection might be explained by the misidentification of the species and the fact that it was not listed in the identification manual (Leslie and Summerell 2006), as assumed by Imathiu et al. (2013a). In contrast to other *Fusarium* species, an infection through FL is nearly symptomless in oats and thus apparently healthy looking grains can contain elevated levels of mycotoxins (Imathiu et al. 2013a).

The pathogenicity of FL was observed *in vitro* using detached leaf assays (Imathiu et al. 2009). Thereby, it was shown that FL is able to be pathogenic to wheat and oat leaves in wounded leaf assays, whereas pathogenicity in unwounded leaves was only observed for oats. Hence, the observations that oats are more often infected with FL and accumulate higher rates of T-2/HT-2 than other cereals, might be due the development of a host preference of FL towards oats. In contrast to other *Fusarium* species, FL was not shown to be a seedling blight pathogen in wheat and oats (Imathiu et al. 2010). Thus, no effect on yield due to foot-rot is assumed and seedling blight seems not to be part of the disease cycle (Imathiu et al. 2010).

The transport via aphids was studied by Drakulic et al. (2016), but no transmission via aphids was observed in wheat. However, higher levels of T-2/HT-2 were measured when aphids were present, suggesting a reduced plant defence.

The results of the above mentioned studies and the symptomless infection explain why the life-cycle of FL is not yet fully understood (Imathiu et al. 2013a), although, Opoku and Edwards (2013) postulated a life cycle with a starting point on infected soil and crop debris (Figure 5).

F. langsethiae produces mainly type-A trichothecenes such as T-2 toxin, its deacetylated from HT-2 toxin, diacetoxyscirpenol and neosolaniol (Kokkonen et al. 2010; Thrane et al. 2004) and in fact, FL has been identified as an important T-2/HT-2 producer in European cereals (Imathiu et al. 2013b; Fredlund et al. 2013; Hofgaard et al. 2016a; Edwards et al. 2012; Edwards et al. 2009). The accumulation of T-2/HT-2 was shown to be favoured by warm and wet conditions before anthesis and dry conditions thereafter (Xu et al. 2013; Langseth and Rundberget 1999).

The toxins T-2 and HT-2 are usually found together and are powerful inhibitors of the protein synthesis (Torp and Langseth 1999). Furthermore, it was reported that they are cytotoxic, cause skin and mucosa erosions, reduction of lymphocytes, affecting the immune defence and growth in exposed animals. During oats processing steps (de-hulling, milling and flaking), a reduction of these toxins occurs, but accompanied by an increase in the resulting by-products that are often used as animal fodder (Pettersson et al. 2011).

The overwintering survival and the source of the main inoculum remain unclear. A further unanswered epidemiological question is the dispersal within the field and onto cereal heads. The occurrence of FL in Swiss cereals and thus the exposition to T-2/HT-2 is unknown. Currently, due to limited knowledge of influencing cropping parameters, there are no recommendations for oat production systems with a reduced risk of T-2/HT-2 contamination.

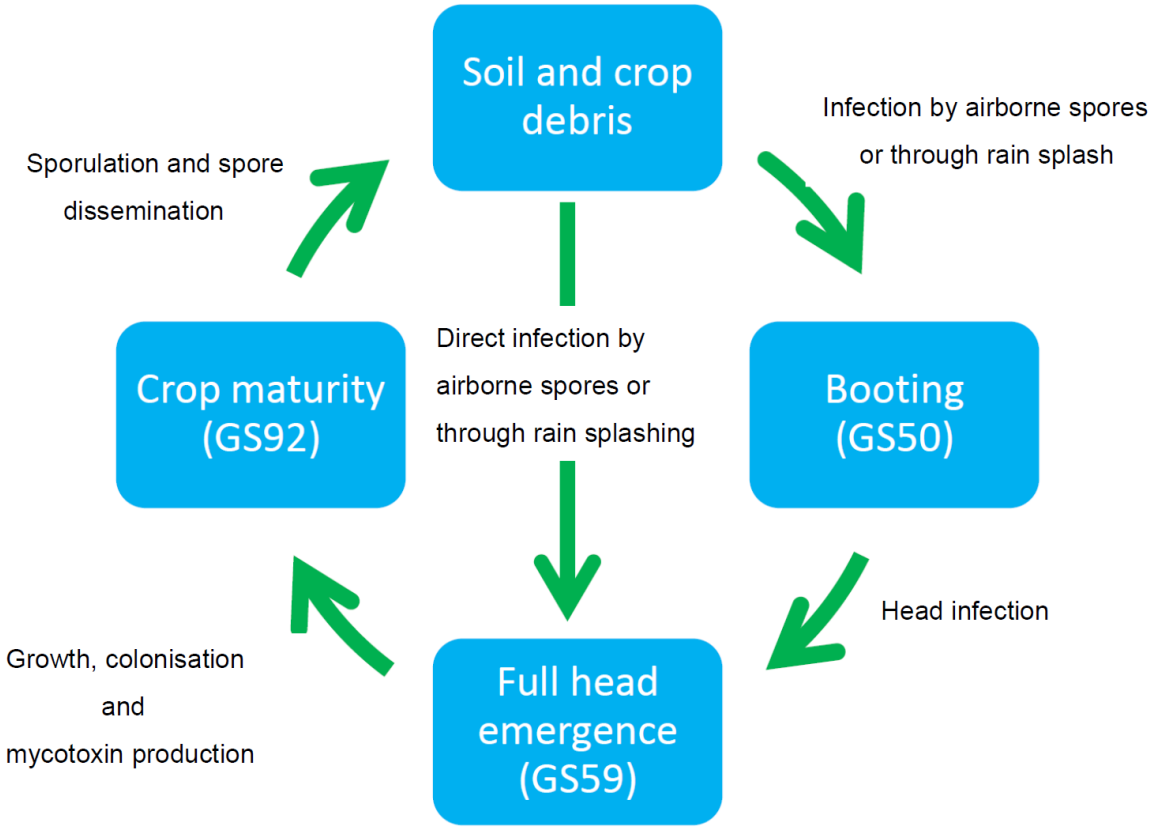


Figure 5: Postulated life-cycle of *Fusarium langsethiae* in cereals (Opoku and Edwards 2013).

1.3 Current measures to control *Fusarium* caused diseases

The control of *Fusarium* species and thus the reduction of mycotoxin contaminated food and feed is necessary for human and animal safety. Thereby, control in the field as well as during storage is necessary. In the field, there are two ways to control *Fusarium* species: one opportunity is the indirect control (prevention) through cultivation measures, the other is the direct control (intervention) with fungicides or biological control agents (Gilbert and Haber 2013). However, no single control measure but a combination of different strategies are needed to efficiently reduce infections through *Fusarium* species.

1.3.1 Agronomic measures

The first agronomic control measure starts before sowing of the cereal with the choice of the previous crop. For FG, various studies have shown that growing a non-host plant reduces the inoculum potential whereas crops such as maize or wheat increase the inoculum on the crop residues (Dill-Macky and Jones 2000; Maiorano et al. 2008; Pereyra and Dill-Macky 2008). In barley and oats, a higher T-2/HT-2 contamination in fields with previous crop small-grain cereal compared with other previous crops was observed in Norway and France (Bernhoft et al. 2012; Orlando et al. 2010).

Furthermore, the reduction of potential inoculum by either burying crop residues via ploughing or chopping of crop residues to accelerate their decomposition was shown to contribute to an FG reduction (Vogelgsang et al. 2011; Blandino et al. 2012; Pereyra and Dill-Macky 2008; Oldenburg et al. 2007). Also, in reduced tillage fields, a higher T-2/HT-2 accumulation in barley and oats was observed compared with that in ploughed fields (Parikka et al. 2007; Edwards et al. 2009; Bernhoft et al. 2012). Still, there is a conflict of interests as 1) reduced tillage has various benefits, such as reduced erosion and leaching, promotion of soil organisms and increase of organic matter but 2) reduced tillage in certain crop rotations has to be regarded as a risk under phytosanitary aspects (Gilbert and Haber 2013; Dill-Macky 2008; Uri et al. 1998).

A further promising measure is the choice of less susceptible varieties. In wheat, it was shown that DON contamination in grains and straw is strongly dependent on the variety (Vogelgsang et al. 2011; Vogelgsang et al. 2009). However, in barley, Nielsen et al. (2014) detected only one variety with significantly less *Fusarium* DNA. In oats, the variety effect is not yet clear since there are contrary results. Suproniene et al. (2010) observed a higher occurrence of FL in

spring than in winter cereals whereas Edwards et al. (2009) observed a higher T-2/HT-2 contamination in winter sown compared with spring sown oats.

A further influencing factor for *Fusarium* species incidence and toxin accumulation is the harvesting time. Generally, a higher incidence of *Fusarium* species (FG and FP) in wheat and barley was observed in samples with delayed harvest, compared with samples from early harvested plots. However, no influence on the DON content was noticed (Xue et al. 2004; Xue et al. 2008; Pageau et al. 2009), but a significantly higher ZEA content was observed in wheat after a later harvest (Kharbikar et al. 2015). Generally, this finding can be explained by the longer colonisation and spreading period.

Another possibility to reduce contaminated cereal grains is the adjustment of the combine harvester. An increasing fan speed and a wider shutter opening reduced the amount of *Fusarium* damaged kernels (FDK) and DON in wheat (Salgado et al. 2014; Salgado et al. 2011). The authors stated that the reduced yield was compensated due to higher prices payed for better grain quality. For FL and FP as well as their main mycotoxins, further studies are needed to identify an influence of harvesting conditions.

The use of nitrogen (N) fertilisation can enhance the susceptibility of cereals to various plant pathogens including *Fusarium* species (Veresoglou et al. 2013). There are contrary results about the influence of N on the presence of *Fusarium* species and mycotoxin accumulation. An increase of disease intensity and DON contamination was observed in wheat fields with N fertilisation above 80 kg ha^{-1} (Lemmens et al. 2004b). Contrary, Yang et al. (2010) noticed a more severe FG infection and higher mycotoxin levels in barley with low N fertilisation (15 kg ha^{-1}) compared with high N fertilisation (100 kg ha^{-1}). A higher N fertilisation increases also the possibility of lodging, which can lead to increased fungal infection and subsequent contamination with DON and NIV in barley and wheat as reported by Nakajima et al. (2008). However, for barley and oats grown in Switzerland, recommendations on how to reduce the risk of *Fusarium* infections with agronomic measures are missing.

1.3.2 Chemical control measures

Application of synthetic fungicides is a common tool to control *Fusarium* species. However, in Switzerland, the utilisation of fungicides against *Fusarium* species during anthesis in barley and generally in oats is not permitted (Hochstrasser 2016). The most commonly chosen fungicide against *Fusarium* species is the group of triazoles leading to lower FHB severity and

DON contents in barley and wheat under natural infection conditions (Jones 2000; Blandino et al. 2012).

The most critical part of fungicide application against FHB is the timing since the main infection period of FG is from the end of ear emergence until the end of flowering (Osborne and Stein 2007). Nevertheless, it was shown that even earlier or late infections can result in disease development and toxin production (Parry et al. 1995; Siou et al. 2014). In most cases, an application is needed when inoculum is present and when favourable weather conditions for *Fusarium* species occur. These are often wet conditions which frequently hinder the trafficability of the fields. An application of various fungicides before flowering to control other fungal diseases increased the *Fusarium* infection in wheat, barley and oats, probably due to the higher suppression of the saprophytic microflora compared with that of the *Fusarium* species (Henriksen and Elen 2005).

For FP and FL, the main infection times are not clear yet. Both species are rather weak pathogens compared with FG and it has been assumed, that they are secondary invaders of the ear (Xu et al. 2005; Audenaert et al. 2009; Divon et al. 2012). For both species, only few studies were done examining the influence of fungicides on growth and toxin production. A reduced NIV content in oats was observed in a field experiment after application of a triazole or a phenpropimorph (Pettersson 1991). In an *in vitro* study with an oat-based matrix, a relative tolerance of FL to tebuconazole compared with prochloraz in terms of growth reduction and toxin production was observed and less effects were seen for phenpropimorph (Kokkonen et al. 2014; Mateo et al. 2011; Mateo et al. 2013).

Apart from fungicides, the application of growth regulators can have an influence on the infection with FHB causing species due to modifications of the crop characteristic. The plants become bushier and shortened, which favours an infection through ejected ascospores and spreading through enhanced humidity (Bernhoft et al. 2012; Maji and Imolehin 2002). However, this holds true only for FG, since the epidemiology of FL and FP is not clear yet.

The application of herbicides should be investigated, since *Fusarium* species were also isolated from weeds (cited in Parry et al. 1995). The application of herbicides, such as glyphosate was shown to increase *Fusarium* incidence and DON content in greenhouse studies (cited in Powell and Swanton 2008). However, this effect was not observed in field trials (Henriksen and Elen 2005; Bérubé et al. 2012). The effect of herbicides on FP and FL incidence is unknown and warrants further studies.

1.3.3 Biological control measures

The reduction of FHB with biological control agents (BCAs) aims to improve the disease control together with the measures described above. The application of BCAs is considered to be less harmful to the environment than synthetic fungicides (Gilbert and Haber 2013; Matarese et al. 2012). Antagonists against *Fusarium* are found among bacteria; (e.g. Palazzini et al. 2017; Jung et al. 2013), yeasts (Zhang et al. 2007; Khan et al. 2004) and fungi; (e.g. Schöneberg et al. 2015; Sarrocco et al. 2013; Xue et al. 2009).

Different modes of action have been observed and the overall control might be the result of several mechanisms working together (Howell 2003; Inch and Gilbert 2011). The reduction of mycotoxins is thought to be the result of direct inhibition, degradation or indirectly via growth reduction (Khan and Doohan 2009; Chan et al. 2009; Dunlap et al. 2011; Jochum et al. 2006).

1.4 Forecasting systems

Disease forecasting systems are based on the combination of weather conditions, host susceptibility and the presence of inoculum (Xu 2003). To develop a reliable forecasting system, the evaluation of several models is needed.

Numerous forecasting systems and models have been developed to prevent FHB, mainly by applying fungicides in wheat focusing on the DON content (Schaafsma and Hooker 2007; Froment et al. 2011; Cowger et al. 2009; Váňová et al. 2009). Most of these models are limited to the weather conditions and only some take into account the agronomic factors. The Swiss forecasting system FusaProg incorporates several factors, including the weather conditions, the growth stage and the respective cropping factors of the field (Musa et al. 2007). Thus, it is not only a tool to decide whether a fungicide is needed but also to rate the effect of the different cropping factors which helps to optimise the farmers cropping system.

Until to date, there is no forecasting system for FHB in barley and oats. Since the 1980s, several models were tested in China to assess the risk of an FHB epidemic in barley, although only for one particular cultivar (cited in Choo 2009). More recently, a model using statistical analysis of both weather and agronomical factors was tested to predict DON in Scandinavian oats (Lindblad et al. 2012) but the authors stated that a prediction was not reliable with the factors investigated, such as flowering and harvesting date, tillage system, previous crop and soil type.

In parallel, no forecasting systems are available for other mycotoxins or other FHB causing species such as FL and T-2/HT-2 or FP and NIV. This is probably a result of the reduced knowledge on the life cycle of the respective fungi.

1.5 The cereals oats and barley - current situation in Switzerland

The agricultural area of Switzerland accounts for approximately 970'000 ha, equalling 23% of the land use, and cereals are grown on 14% (144'000 ha) of this area (Federal Statistical Office 2017c). The total cereal production in 2015 was 891'000 t, thereof 539'000 t (60%) were produced for human consumption and 352'000 t (40%) for fodder. The main cereals used for human consumption were wheat, spelt and rye, with a crop area proportion of 53%, 3% and 1%, respectively. Other cereals such as emmer, sorghum, oats and barley accounted together for only 0.2%. In 2014, the level of self-sufficiency with cereals for human consumption was 53% (Federal Statistical Office 2017a). The main cereals used for fodder were barley, maize, triticale, wheat and oats, with a crop area proportion of 19%, 10%, 6%, 4% and 1%, respectively (Federal Statistical Office 2017a). In 2014, 56% of the produced and imported cereals were used for fodder whereas 37% were used for human consumption (Federal Statistical Office 2017a).

Overall, the area used for cereals production is in decline since the last 30 years. In 1985, 184'000 ha were used for cereal production, including 93'000 ha for wheat, 50'500 ha for barley and 10'000 ha for oats. In 2016, 145'000 ha were used for cereal production which equals a decline of 20%. The trend is the same for the production area of wheat 84'000 ha (-10%), barley 29'000 (-43%) and a dramatic decline of oats 1'700 (-83%) (Federal Statistical Office 2017b). However, in contrast to the decreasing production area, the yield increased within the last 50 years in all three above mentioned crops: In wheat the mean yield in 1965, 1985 and 2014 was 3.2, 5.7 and 6.2 t ha^{-1}, respectively. For barley and oats the yield in 1965, 1985 and 2014 was 3.1 and 2.9 t ha^{-1}, 5.3 and 5.2 t ha^{-1}, and 7.4 t and 5.3 t ha^{-1}, respectively (Food and Agriculture Organization of the United Nations 2017).

The main countries for import of soft and hard wheat used as fodder or for human consumption, as well as of barley for human consumption are Germany, France and Italy. Fodder barley is mainly imported from Germany, France and the Czech Republic. Malting barley is only imported from Germany. Oats are imported mainly from Finland, Germany, the Czech Republic

and France, either as fodder, for human consumption or rolled oats (Federal Customs Administration 2017).

1.6 Health benefits of barley and oats

In addition to wheat, less grown cereals such as barley and oats frequently contain a broader spectrum of health promoting compounds, including flavonoids, phenolic acids and β-glucans which help to improve the nutritional balance of consumers (Dykes and Rooney 2007; Peterson 2001; Brennan and Cleary 2005; Mazza and Miniati 1993). One group of phenolics, the avenanthramides, are exclusively present in oats (Collins 1989; Hüseyin 2015).

All these compounds are secondary metabolites, which evolve from the shikimate-phenylpropanoid-flavonoid pathway. They belong to structurally diverse chemical classes and are produced for multiple purposes such as pigmentation, reproduction or resistance to plant pathogens (Lattanzio et al. 2006). Generally, phenolic compounds are categorised into the groups of non-flavonoid and flavonoid phenylpropanoids and are the major contributors to the antioxidant activity of cereals (Atanasova-Penichon et al. 2016; Boutigny et al. 2008).

Moreover, these compounds are associated with health benefits including the control of the cholesterol level, prevention of atherosclerosis and attenuation of insulin levels (Anttila et al. 2004; Sharma and Kotari 2017; Liu et al. 2004). Furthermore, they have an antioxidant potential and are considered to prevent cancer, cardiovascular diseases and Parkinsons, by scavenging free radicals and thereby decreasing oxidation (Li et al. 2005; Zhou et al. 2004; Adom and Liu 2002; Brindzová et al. 2008; Martínez-Villaluenga and Peñas 2017). These compounds are mainly located in the outer layers of the grain (Abdel-Aal and Hucl 2003; Dykes and Rooney 2007), hence, the consumption of cereal bran or whole cereal flours is recommended for a healthy diet.

Still, the concentration of health promoting compounds can be influenced by prevailing weather conditions and in response to pathogens (Niemann 1993; Chappell et al. 2017). With respect to the impact of environmental conditions, several studies showed that high precipitation and colder temperatures reduce the amount of β-glucans, phenolic acids and dietary fibres (Ma et al. 2015; Ehrenbergerová et al. 2008; Chappell et al. 2017). The first report of phenolics providing diseases resistance was shown in onions (*Allium cepa* L.) preventing onion smudge diseases (*Colletotrichum circinans* Berk, Voglino) (cited in Lattanzio et al. 2006).

Moreover, the antifungal properties of some phenolic acids against the growth and mycotoxin production of different *Fusarium* species was shown in several *in vitro* studies (Gauthier et al.

2016; Ponts et al. 2011; Boutigny et al. 2009; Ferruz et al. 2016). However, some phenolic acids also enhanced the mycotoxin production in these studies. Further, the exact mechanism of the antifungal activity is not yet fully understood but it is hypothesised, with respect to toxigenic species, that free radicals are bound, which reduces oxidative stress and thus limits the mycotoxin production (Atanasova-Penichon et al. 2016).

Hence, cultivating barley and oat varieties with high contents of health promoting compounds and with adjusted cropping techniques can 1) help to produce cereals for human consumption, which are free of mycotoxins and 2) enhance the health of consumers. Additionally, the production in Switzerland would increase the level of self-sufficiency and would also be more sustainable due to reduced transport costs.

1.7 Objectives and Outline

Knowledge about the disease triangle, including the plant, the pathogen and the environment, are fundamental to develop and recommend cropping techniques to prevent diseases. Previous studies contributed to a valuable understanding of FG in wheat and maize but less was done in minor grown cereals such as barley and oats.

This study was conducted to 1) identify both the *Fusarium* species and mycotoxin spectra in barley and oats cultivated in Switzerland; 2) with knowledge about these spectra, the investigation of cropping measures, which influence the occurrence of *Fusarium* species and mycotoxin accumulation was done. Thereby, recommendations for farmers could be established which reduce the risk of contamination of barley and oats with mycotoxins (chapters 2 and 3). For this, a Swiss wide survey with commercial oat and barley samples was performed.

A further aim of this study was to 3) gain an improved understanding of the epidemiology of the dominant *Fusarium* species in oats and barley. For this, studies about the dispersal in the field and influence of weather conditions on the infection were performed. Specific temperature and humidity durations in the climate chamber were tested to examine the effect on the infection and mycotoxin production (chapters 4 and 5).

This fundamental knowledge is needed for an integrated control of *Fusarium* species in barley and oats and to extend the forecasting system FusaProg. Ultimately, results of this study will provide a contribution to reduced fungicide applications and the cultivation of mycotoxin free cereals.

2 Fusarium and mycotoxin spectra in Swiss barley are affected by various cropping techniques

published in Food Additives and Contaminants: Part A

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2.1 Abstract

Fusarium head blight is one of the most important cereal diseases worldwide. Cereals differ in terms of the main occurring *Fusarium* species and the infection is influenced by various factors, such as weather and cropping measures. Little is known about *Fusarium* species in barley in Switzerland, hence, harvest samples from growers have been collected in 2013 and 2014, along with information on respective cropping factors. The incidence of different *Fusarium* species was obtained by using a seed health test and mycotoxins were quantified by LC-MS/MS. With these techniques, the most dominant species, *F. graminearum*, and the most prominent mycotoxin, deoxynivalenol (DON), were identified. Between the three main Swiss cropping systems, Organic, Extenso and Proof of ecological performance, we observed differences with the lowest incidence and toxin accumulation in organically cultivated barley. Hence, we hypothesise that this finding was based on an array of growing techniques within a given cropping system. We observed that barley samples from fields with maize as previous crop had a substantially higher *F. graminearum* incidence and elevated deoxynivalenol accumulation compared with other previous crops. Furthermore, the use of reduced tillage led to a higher disease incidence and toxin content compared with samples from ploughed fields. Further factors increasing *Fusarium* infection were high nitrogen fertilisation as well as the application

of fungicides and growth regulators. Results from the current study can be used to develop optimised cropping systems that reduce the risks of mycotoxin contamination.

2.2 Introduction

Fusarium head blight (FHB) is one of the most noxious diseases in cereals and is caused by a complex of different *Fusarium* species (Parry et al. 1995); with *F. graminearum* (SCHWABE; teleomorph *Gibberella zeae* SCHWEIN, (PETCH)) being the predominant species in wheat in Switzerland and worldwide (Parry et al. 1995; Vogelgsang et al. 2011). FHB causing species are known to produce different mycotoxins, amongst others trichothecenes (deoxynivalenol (DON), nivalenol, T-2 toxin and HT-2 toxin) or the mycoestrogen zearalenone which threaten human and animal health (Desjardins 2006). In 2006, the European Commission set maximum limits of 1'250 µg kg^{-1} deoxynivalenol and 100 µg kg^{-1} zearalenone for unprocessed cereals for human consumption and established guidance values for feedstuff (The European Commission 2006), which were adopted in Swiss legislation.

Although frequently isolated from symptomatic ears together with *Fusarium* species, *Microdochium nivale/M. majus* ((FRIES) SAMUEL and HALLET; teleomorph *Monographella nivalis* (SCHAFFNIT) MÜLLER) is a non-toxigenic FHB causing species (Nielsen et al. 2014). The species complex responsible for Fusarium head blight in Switzerland was studied in wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) (Vogelgsang et al. 2009; Dorn et al. 2009), but only little is known about the occurrence of *Fusarium* species in Swiss barley (*Hordeum vulgare* L.).

Barley is grown either as fodder (winter barley), for malt and beer production (spring barley) as well as for baking and cooking purposes. Infections with *Fusarium* species and thus accumulation of mycotoxins is harmful in all uses. Fodder barley contaminated with deoxynivalenol, can result in feed refusal, diarrhoea and vomiting in farm animals (D'Mello et al. 1999). In addition, although not acutely toxic, the estrogenic effect of zearalenone can severely disrupt the reproductive system of animals (D'Mello et al. 1999). The contamination of barley for human consumption may also cause problems, since mycotoxins frequently remain in the final product (Malachova et al. 2012).

There are several approaches to reduce the risk of *Fusarium* mycotoxins in cereals: cropping measures (Blandino et al. 2012), biocontrol with microorganisms (Schisler et al. 2002) and fungicides (Haidukowski et al. 2012). However, no single approach but an integrated pest management can sufficiently reduce the risk of *Fusarium* infections.

Switzerland has three production systems that differ in terms of production methods and intensity. (1) The ÖLN system (Ökologischer LeistungsNachweis = proof of ecological performance (PEP)) is the minimum standard for an environmentally friendly agriculture to obtain direct payments. Some of the most important requirements are: balanced use of fertilisers, strict crop rotation, appropriate measures for soil protection and selective application of plant protection products. (2) The Extenso system renounces additionally the use of growth regulators, fungicides, insecticides and synthetic stimulators of natural defence mechanisms. (3) The Organic system completely prohibits the use of synthetic plant protection products and mineral fertilisers. The cultivation of barley is possible in all three systems and the modification of various factors within the respective cropping system, e.g. choice of variety, tillage, crop rotation, fertilisation and many more can influence the diseases pressure, especially for *Fusarium* species (Bernhoft et al. 2012).

Therefore, the objective of this two year monitoring was to gain fundamental knowledge about the occurrence of *Fusarium* species and their associated mycotoxin spectrum in commercial barley samples in Switzerland and to determine and quantify the most influencing cropping techniques to reduce FHB infection.

2.3 Material & Methods

Collection of samples and identification of *Fusarium* species

With support of the cantonal plant protection officers, several hundred farmers were addressed to send a sample of their barley harvest together with the respective agronomic data (see Table 9 in the supplementary material (chapter 2.8)). These data were for example: type of cropping system, variety, previous crop, pre-previous crop and tillage.

In 2013, 280 samples from 18 cantons and in 2014, 160 samples from 17 cantons were obtained. Samples were collected after harvest directly from the combine, from trailers leaving the field or from the silo. To ensure a representative sample, all participating growers received written instructions (see Table 9 in the supplementary material (chapter 2.8)) on how to take ten subsamples and mix these to a sample of approximately 1 kg. The growers sent their samples in plastic bags within one day to Agroscope, Zurich-Reckenholz, along with a completed questionnaire on agronomic data pertaining to the particular field sample. Upon receipt, the samples (ø 12% rel. humidity) were transferred into paper bags and stored at 10 °C until further processing.

In order to obtain representative subsamples, the total sample was processed using a riffle divider (Schieritz & Hauenstein AG, Arlesheim, Switzerland*). Fusarium* species incidence (percent infection) was determined (approximately 6 g equivalent to 100 grains), using an seed health test as described by Vogelgsang et al. (2008b), and 150 g of the respective subsamples were taken for mycotoxin analysis. The different *Fusarium* species were identified according to Leslie and Summerell (2006). Samples of 150 g each were ground with a grain mill (Cyclotec 1093 sample mill, Foss Tecator, Höganäs, Sweden), using a 1 mm screen and the flour was stored at -20 °C until analysis.

Mycotoxin analysis

All ground samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the content of Type A and B trichothecenes: nivalenol (NIV), DON, fusarenon-X (FUS-X), acetyldeoxynivalenol (AcDON), neosolaniol (NEO), diacetoxyscirpenol (DAS), T-2 toxin (T-2), HT-2 toxin (HT-2) and for zearalenone (ZEA). A reference sample of naturally DON-contaminated barley (Trilogy, Washington, MO, USA) was included in each run as method control.

For mycotoxin extraction, 10 g of flour was suspended in 40 ml extraction solution (acetonitrile/acetone/Milli-Q® water (50:25:25)) (acetonitrile and acetone from Scharlau Multisolvent®, Sentmenat, Spain; Milli-Q® water was produced with a Milli-Q® gradient A10 water purification system from Millipore, Bedford, MA, USA) and shaken for 90 min at 175 rpm on a rotary shaker. The suspension was filtered through a Whatman® 595 ½ 150 mm filter (GE Healthcare UK Limited, Buckinghamshire, Little Chalfont, UK).

Extract cleaning was performed by using 3 ml solid phase extraction tubes, fitted with 20 µm frits (Biotage, Uppsala, Sweden) at the bottom. A mixture of 0.15 mg Alox:Celite (50:50; Alox activated 6 h by 400 °C) was weighed into the tube and closed with an additional frit. The cartridges mounted on a Visiprep Solid Phase Extraction Vacuum Manifold (12-port-model) (Supelco, Bellefonte, PA, USA) were washed with 2 ml of the extraction solution. Extracts were filtered through the cartridges and thereafter the solid phase was washed with 2 ml of extraction solution. The remaining solvent in the tubes was drawn into 5 ml conical reaction vessels (Supelco) under vacuum.

The eluate was concentrated by a gentle airstream at maximal 50 °C. The concentrated solvent (approximately 400 µl) was then transferred into a 1.5 ml HPLC-Vial by a Pasteur pipette. 400 µl water-methanol (90:10) was added into the collection vial, vortexed and combined in

the HPLC-vial which was then volumetrically adjusted with water-methanol (90:10) to a volume of 1 ml.

LC-MS/MS was performed with a Varian 1200L LC-MS System (VarianInc, Walnut Creek, CA, USA). Separation of the trichothecenes was performed by using a Polaris Amide C18-A column (3 µm, 50 x 2.0 mm, VarianInc) and a guard column (SecurityGuard™ Cartridge C18 4 x 2.0 mm, Phenomenex Inc, Torrance, CA, USA). Eluent A consists of Milli-Q® water/methanol (95/5 v/v) and eluent B of Milli-Q® water/methanol (5/95 v/v). Both eluents contained 5 mM ammoniumacetate. The mobile phase flow rate was 0.25 ml min^{-1}. Detection of the trichothecenes was performed with the APCI-interface using negative and positive modes and ions. The interface parameters and gradient elution of the LC-MS/MS are shown in Table 1.

The method was validated by recovery experiments of differently spiked samples (200 and 2500 µg kg^{-1}), respectively. The recovery range for each trichothecene was between 85 and 120%. The limit of quantification (LOQ) was determined as ten times the baseline noise. The limit of detection (LOD) was determined as three times the baseline noise. The range of LOQ and LOD are shown in Table 2. The LOQ and LOD vary because the samples were measured in different runs.

Table 1: Interface parameters and gradient elution of the LC-MS/MS in positive and negative mode used for mycotoxin analysis.

Parameter	negative mode	positive mode
Corona	- 12 V	+ 7.5 V
Shield voltage	- 600 V	+ 600 V
Housing temperature	50 °C	50 °C
Drying gas	18.2 psi, 310 °C	18.2 psi, 310 °C
Vaporize gas temperature	12.7 psi, 275 °C	12.7 psi, 275 °C
Nebulising gas	45.1 psi	45.1 psi
CID collusion gas pressure	1.8 mTorr	1.8 mTorr
Detector voltage	1800 V	1200 V
Gradient elution	0.0 min 5% B	0.0 min 20% B
	1.0 min 5% B	0.5 min 45% B
	4.0 min 30% B	5.5 min 75% B
	5.0 min 100% B	6.0 min 100% B
	9.5 min 100% B	9.0 min 100% B
	10.0 min 5% B	9.5 min 20% B
	15.0 min 5% B	15.0 min 20% B

Table 2: Ranges of limit of quantification (LOQ) and limit of detection (LOD).

Mycotoxin	LOQ	LOD
DON	40-72	12-22
NIV	15-38	4-11
ZEA	6-40	2-12
T-2	34-72	10-22
HT-2	4-9	1-3

Statistical analysis

Statistical analysis was conducted with ʻRʼ version 3.1.0 (R Core Team 2015) and ʻR Studioʼ version 0.98.994 (R Studio Team 2015). The package ʻagricolaeʼ (de Mendiburu 2015) was used to calculate Pearson's correlation, the package 'userfriendlyscience' (Peters 2016) for the Games-Howell post-hoc test and the package 'multcomp' (Hothorn et al. 2008) for the Tukey-Kramer post-hoc test. Modell selection was done by the aid of the packages ʻMuminʼ (Barton 2016) and ʻnlmeʼ (Pinheiro et al. 2016).

Homogeneity of variance and normality of residuals were checked graphically using plots of fitted values versus the root of the standardised residuals and normal Q-Q plot, respectively. To meet homogeneity of variance and normal distribution of residuals, incidence of *F. graminearum* data and DON concentrations were arcsin (square root) and log transformed, respectively. Mycotoxin concentrations below the respective LOQ or LOD were calculated as LOQ/2 or LOD/2, respectively.

To determine influencing factors on *F. graminearum* incidence and DON concentration, a multiple linear regression model was employed using stepwise backward/forward regression. The obtained regression models were evaluated by using the Akaike Information Criterion (AIC). All factors were checked for multi-collinearity using a correlation matrix, factors with a correlation coefficient > 0.3 were removed from the model. The model with the greatest explanation rate was used for further analysis.

Significant influencing factors were further analysed with the Games-Howell test, whereas interactions of the factors previous crop x additional chopping, previous crop x tillage and previous crop x pre-previous crop were analysed with the Tukey-Kramer test. Data analysis was performed with a probability value of 5% ($\alpha = 0.05$). The correlation between the DON concentration and the incidence of *F. graminearum* were calculated using Pearson's correlation coefficient (r) on transformed data (see above).

The co-existence of species of the FHB complex and the respective mycotoxins was explored using a Principal Component Analysis (PCA) on the correlation matrix of 14 variables. These

variables were incidence of different *Fusarium* species (%) of *F. graminearum*, *F. avenaceum*, *F. poae*, *F.* spp, *Microdochium* spp. and mycotoxin content (µg kg^{-1}) of DON, NIV and ZEA. For figures and tables, untransformed data were used. Figures were created with 'R' version 3.1.0 (R Core Team 2015) and 'R Studio' version 0.98.994 (R Studio Team 2015) and Microsoft® Excel 2013.

2.4 Results

Due to the overall low occurrence of *Fusarium* species and mycotoxins, the analysis on potential cropping factors was done only for the main occurring species/mycotoxin, *F. graminearum* and DON, and pooled over both years, since the influencing factors were the same in both years. In order to obtain meaningful sample sizes, cropping factors were grouped as follows: barley varieties were Caravan, Cassia, Franziska, Fridericus, Landi, Meridian, Quench, Semper, Zoom or Other (12 different varieties with each fewer than 15 samples); previous crops were cereals (barley, oats, spelt, emmer, triticale, wheat), maize, pasture, canola or other (12 different crops with each fewer than 15 samples); pre-previous crops were cereals (barley, oats, spelt, emmer, triticale, wheat), maize, pasture, canola, sugar beet or other (16 different crops with each fewer than 10 samples); additional chopping was yes or no; tillage was plough or reduced tillage (including direct sowing); fungicide ingredient were triazoles + strobilurins, strobilurins only, triazoles only or no fungicide; applied nitrogen (N) in kg ha^{-1} were 1 = 1-50 kg N ha^{-1}, 2 = 51-100 kg N ha^{-1}, 3 = 101-150 kg N ha^{-1}, 4 = 151-200 kg N ha^{-1} or 5 >200 kg N ha^{-1}. If the N amount kg ha^{-1} was not indicated, it was calculated according to averaged values for farmyard manure.

Fusarium species spectrum in Swiss barley samples from 2013 and 2014

Overall, nine different *Fusarium* species were detected in Swiss barley samples. These were, in descending order, *F. graminearum* (FG), *F. avenaceum* (Fries, Saccardo; teleomorph *Gibberella avenacea*, Cook), *F. poae* (Peck, Wollenweber; no teleomorph known), *F. culmorum* (Smith, Saccardo; no teleomorph known), *F. crookwellense* (Burgess, Nelson & Toussoun; no teleomorph known), *F. dimerum* (Penzig; no teleomorph known), *F. equiseti* (Corda, Saccardo; teleomorph: *Gibberella intricans* Wollenweber), *F. tricinctum* (Corda, Saccardo; teleomorph: *Gibberella tricincta* El-Gholl, McRitchie, Schoulties & Ridings) and *F. sporotrichioides* (Sherbakoff; no teleomorph known).

Fusarium graminearum was the predominant species in 2013 (62% of all detected *Fusarium* species) and 2014 (45%), whereas *F. avenaceum* was the second most occurring species with 29% and 33%, respectively. *Fusarium poae* was the third most frequent species with 4% and 17%, respectively. Other *Fusarium* species occurred with one percent or lower (Figure 6). The non-toxigenic species *Microdochium nivale/M. majus* were the most occurring FHB causing species in both years (74%, 69%). However, due to their inability to produce mycotoxins, the corresponding data were not included in Figure 6. The samples with the highest occurrence of *F. graminearum* in 2013 and 2014 showed infection rates of 53% and 25%, respectively. However, the average incidence of the three predominant *Fusarium* species was relatively low (<5%) in both years (Table 3) but with a higher infection rate in 2013 (3.8%) compared to 2014 (2.4%) (Figure 7).

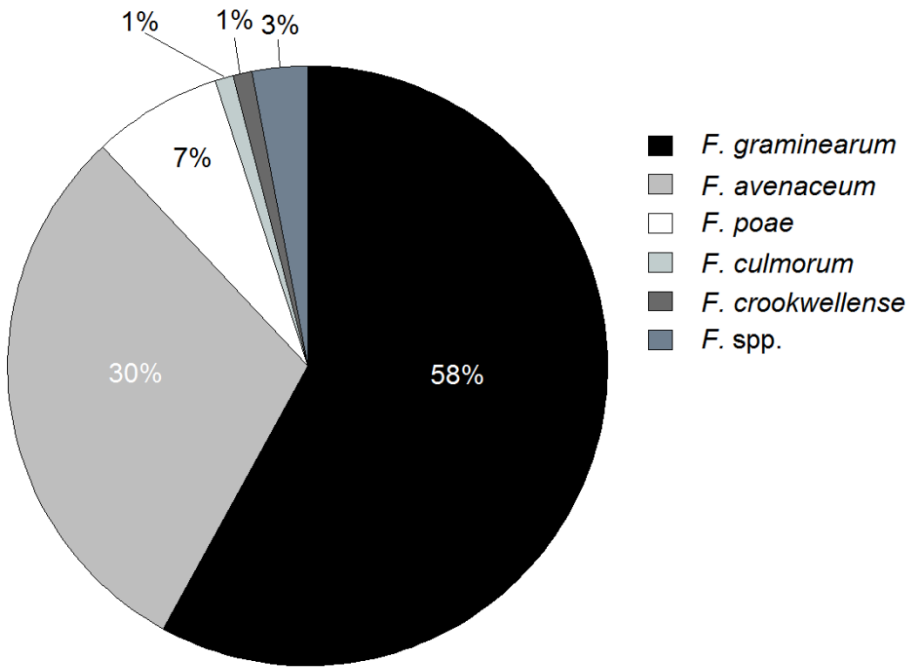

Figure 6: Ratio of different species from all detected *Fusarium* species in Swiss barley samples collected in 2013 and 2014; *F.* spp.: *Fusarium* species with an incidence of < 1%; number of samples = 440.

Table 3: Mean incidence of *Fusarium graminearum* (FG), *F. avenaceum* (FA) and *F. poae* (FP) in Swiss barley samples collected in 2013 and 2014. n = number of samples.

Year	n	Mean incidence of *Fusarium* species (%) ± 95% confidence interval		
		FG	FA	FP
2013	280	3.8 ± 1.8	1.8 ± 0.3	0.2 ± 0.1
2014	160	2.4 ± 1.3	1.0 ± 0.3	1.9 ± 0.2

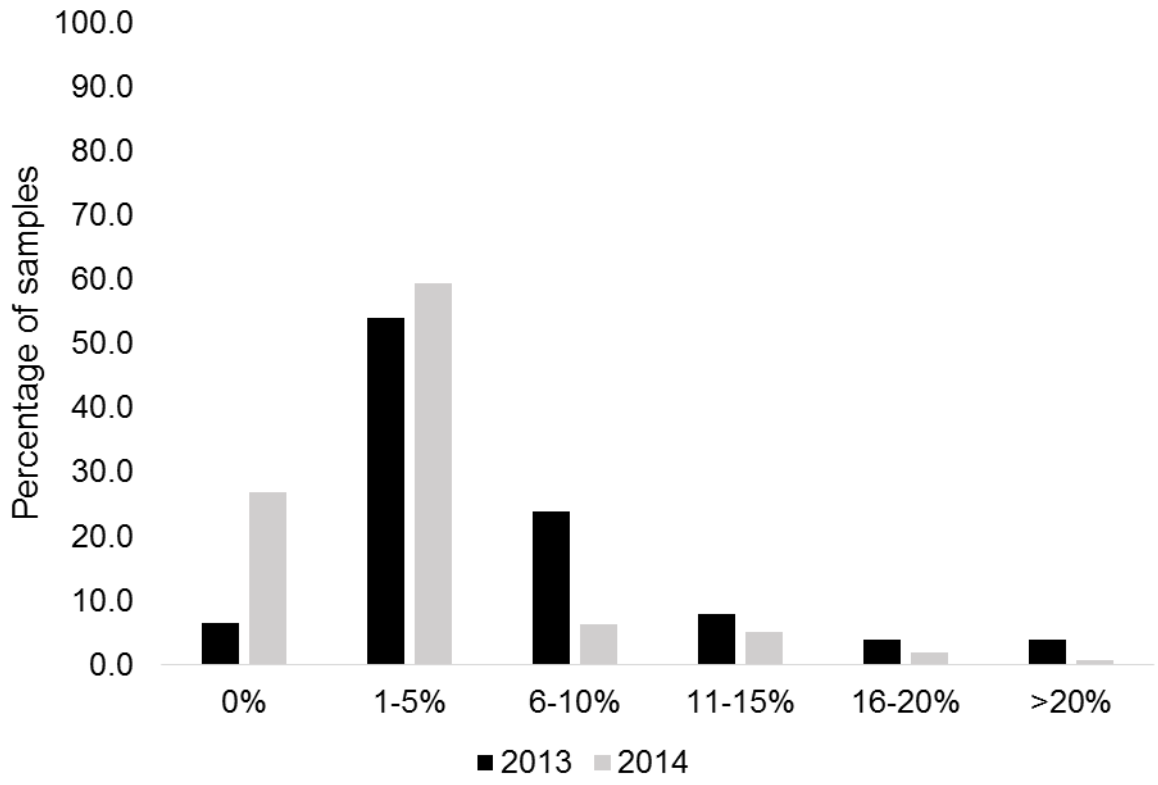

Figure 7: Number of Swiss barley samples collected in 2013 (n = 280) and 2014 (n = 160) within infection classes (%) of all detected *Fusarium* species; black bars = harvest 2013, grey bars = harvest 2014.

Fusarium mycotoxins in Swiss barley samples from 2013 and 2014

The mycotoxin quantification via LC-MS/MS revealed DON as the predominant mycotoxin in 2013 and 2014 with an average of 235 µg kg^{-1} and 47 µg kg^{-1}, respectively. The contamination with DON and T-2/HT-2 was higher in 2013, whereas the contamination with NIV and ZEA was higher in 2014 (Table 4). The European Union maximum limit for human consumption (The European Commission 2006) of DON of 1'250 µg kg^{-1} for unprocessed barley was only

exceeded in nine samples (3%) in 2013 and in one sample (<1%) in 2014. The highest measured DON contents were 4'860 µg kg^{-1} in 2013 and 1'725 µg kg^{-1} in 2014. The maximum limit of 100 µg kg^{-1} ZEA in unprocessed cereals was exceeded only in 2014 by three samples (2%).
The toxins T-2/HT-2 were detected in 16 (6%) samples in 2013 and in ten (6%) samples in 2014. The proposed indicative limit of 200 µg kg^{-1} in unprocessed barley (The European Commission 2013) was exceeded only in 2013 in two (<1%) samples.

Table 4: Deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA) and T 2/HT 2 toxins in Swiss barley samples collected in 2013 and 2014 described by mean value, 95th confidence interval, maximum detected value and number of samples with mycotoxins below the limit of detection. n = number of samples.

Year	n	Mean mycotoxin concentration (µg kg^{-1}) ± 95% confidence interval (Maximum detected value) Percentage of samples below LOD			
		DON	NIV	ZEA	T-2/HT-2
2013	280	240 ± 56 (4860) 17	13 ± 4 (435) 74	4 ± 1 (84) 79	15 ± 1 (319) 94
2014	160	47 ± 24 (1725) 69	31 ± 8 (431) 94	10 ± 4 (240) 86	10 ± 2 (155) 94

LOQs (limit of quantification) (µg kg-1): DON = 40-72, NIV = 15-38, ZEA = 6-40, T-2 = 34-72, HT-2 = 4-9
LODs (limit of detection) (µg kg-1): DON = 12-22, NIV = 4-11, ZEA = 2-12, T-2 = 10-22, HT-2 = 1-3.
Mycotoxin concentrations below the respective LOQ or LOD were calculated as LOQ/2 or LOD/2, respectively.

Although the contamination with mycotoxins was low in both years, a significant positive correlation ($r = 0.72$, $p < 0.001$) was found between DON content and FG incidence. In addition, a significant positive relationship between *F. poae* incidence and nivalenol content ($r = 0.60$, $p < 0.001$) was observed.
Fusarium species incidence and mycotoxin content were plotted as a biplot (Figure 8) to show the distribution of the samples in the two most descriptive dimensions of the descriptive factors and the variables (species and mycotoxins) projected onto these two axes. On the x and the y axis, factor 1 and 2 describe 26.2% and 19.9% of the variability, respectively. There was a strong positive relationship between *F. poae* and NIV and between FG, DON and ZEA. In addition, there was also a positive relationship between FG and *Microdochium* spp., while *F. avenaceum* showed a positive relationship with *F.* spp, except *F. poae* and FG. In contrast, there was a strong negative relationship between *Microdochium* spp. and *F. poae* and *F. avenaceum.*

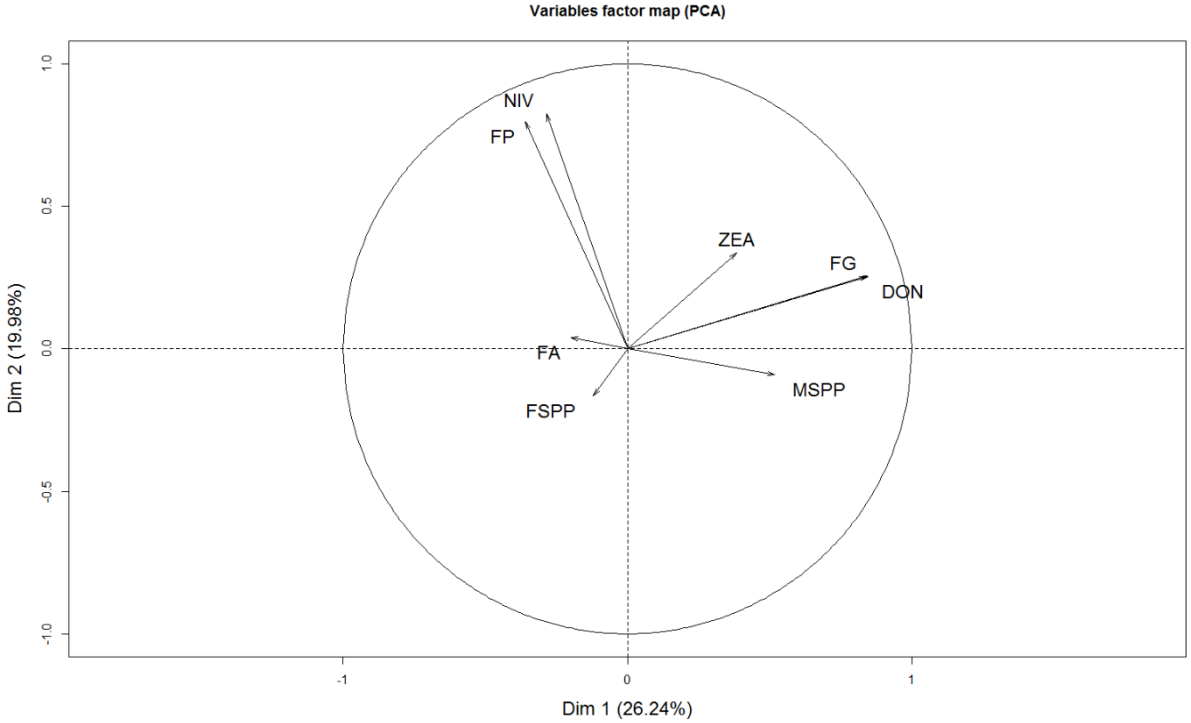

Figure 8: Biplot of the principal component analysis of *Fusarium* species incidence (%) with *F. graminearum* (FG), *F. avenaceum* (FA), *F. poae* (FP), *F.* spp. (FSPP), *Microdochium* spp. (MSPP) as well as the mycotoxin content (µg kg^{-1}) with deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) in Swiss barley samples collected in 2013 and 2014, FSPP: *F. culmorum*, *F. crookwellense*, *F. equiseti*, *F. tricinctum*, *F. dimerum* and *F. sporotrichioides*, n = 440.

Cropping system

In both years, differences were found between the three main cropping systems in Switzerland: Organic (n = 42), Extenso (n = 173) and PEP (n = 225). Due to multicollinearity this factor was removed from the model and thus no statistical analysis was done. In 2013 and 2014, the samples from the PEP system showed a higher mean incidence of FG (5.4%, 4.1%) and a higher mean contamination with DON (324.4 µg kg^{-1}, 78.3 µg kg^{-1}), respectively, compared with the other cropping systems. Samples from the organic system had the lowest mean FG incidence (0.6%, 0.4%) and contamination with DON (21.8 µg kg^{-1}, 26.0 µg kg^{-1}), respectively.

Variety

Differences between the grown barley varieties have been observed. Due to multicollinearity, this factor was removed from the model and thus no statistical analysis was performed. In winter varieties, the mean FG incidence (3.1%) and DON content (179.5 µg kg^{-1}) were higher compared with spring varieties (0.8%; 39.1 µg kg^{-1}). For example, the spring variety Quench

showed the lowest incidence (0.6%) and DON content (30.0 µg kg^{-1}), whereas the winter varieties Meridian and Zoom showed the highest FG incidences (5.3%, 3.7%) and DON contents (300.0 µg kg^{-1}).

Crop rotation and tillage

The cultivation of maize before barley resulted in a significantly higher ($p<0.001$) mean incidence of FG (7.3%) and DON content (447.7 µg kg^{-1}) compared with other previous crops except canola. From the Organic, Extenso and PEP cropping systems, maize as a previous crop was grown by 14%, 8% and 20%, respectively. The lowest average incidence (0.8%) and DON content (31.1 µg kg^{-1}) was noticed when the previous crop was pasture (Table 5). From the Organic, Extenso and PEP cropping systems, pasture as a previous crop was grown at 36%, 6% and 1%, respectively. A reduction ranging from 48% to 89% FG incidence and 53% to 93% DON content was obtained when maize was not the previous crop (Table 5).

Table 5: Effect of previous crop on *Fusarium graminearum* (FG) incidence and DON content in Swiss barley samples, collected in 2013 and 2014. n = number of samples; SEM = standard error of mean. Means with the same letters are not significantly different according to a Games-Howell test at $\alpha = 0.05$. Details about previous crops as explained in the main text.

Previous Crop	n	FG incidence (%)	DON (µg kg^{-1})
		Mean (SEM)	
Maize	64	7.3 (1.2) a	448 (102) a
Canola	28	3.8 (0.9) ab	210 (64) ab
Other	41	2.6 (0.9) bc	154 (35) ab
Cereal	280	2.1 (0.2) bc	110 (14) b
Pasture	27	0.8 (0.3) c	31 (8) c

Overall, samples from fields with ploughed soils showed significantly ($p < 0.001$) less FG incidence and DON content compared with samples from reduced tillage fields regardless of the previous crop (Table 6). The reduction of FG incidence and DON content by ploughing was 47% and 29%, respectively. In fact, 95% of the organic farmers ploughed the soil before growing barley in contrast to 64% (Extenso) and 58% (PEP). Furthermore, samples from ploughed fields showed significantly lower FG incidence if the previous crop was a cereal ($p = 0.046$) or maize ($p < 0.001$) compared with samples from reduced tillage fields with the same previous crops.

Maize as a previous crop in combination with reduced tillage showed the highest mean incidence of FG (9.8%) and DON content (448.5 µg kg^{-1}) (Table 6). All organic farmers ploughed the soil after growing maize, compared with 12% of the Extenso and 53% of the PEP farmers.

A significantly ($p < 0.001$) lower DON content was only observed in samples from ploughed fields with previous crop cereals compared with samples from cereal fields with reduced tillage (Table 6). A total of 96% of the organic, 71% of the Extenso and 65% of the PEP farmers ploughed their fields after growing cereals.

Table 6: Effect of tillage and the interaction of previous crop x tillage on *F. graminearum* (FG) incidence and DON content in Swiss barley samples collected in 2013 and 2014. n = number of samples; SEM = standard error of mean. Means with the same letters are not significantly different according to a Games-Howell test and a Tukey-Kramer test for interactions both at $\alpha = 0.05$. Meanings in cropping factors as explained in the main text.

Cropping factor	n	FG incidence (%)	DON (µg kg-1)
Tillage		Mean (SEM)	
Reduced tillage	160	4.2 (0.5) a	203 (26) a
Plough	280	2.2 (0.3) b	143 (25) b
Previous crop + tillage		Mean (SEM)	
Maize + reduced	33	9.8 (1.6) a	449 (100) a
Maize + ploughed	31	4.6 (1.8) bc	447 (184) ab
Canola + ploughed	13	3.8 (1.4) abc	281 (128) abc
Canola + reduced	15	3.7 (1.1) bc	147 (45) abcd
Other + ploughed	20	3.0 (1.7) bc	181 (60) abcd
Cereal + reduced	86	2.8 (0.4) b	146 (22) b
Other + reduced	21	2.3 (0.7) bc	129 (38) bcd
Cereal + ploughed	194	1.8 (0.3) c	94 (17) cd
Pasture + reduced	5	1.2 (0.8) bc	26 (14) bcd
Pasture + ploughed	22	0.7 (0.3) bc	32 (10) d

Additional chopping of previous crop residues showed also an effect on FG incidence and DON content. Chopping maize residues significantly reduced mean FG incidence ($p = 0.013$) and DON content ($p = 0.017$) compared to non-chopped maize residues. The resulting reduction with previous crop maize was 53% in terms of incidence and 42% in terms of DON content. This effect was not observed in any of the other previous crops (data not shown). This additional treatment was carried out by 83% of the organic farmers, compared with 57% of the Extenso and 25% of the PEP farmers.

The crop rotation two years before barley showed a higher FG incidence when maize was grown after maize and this combination was significantly different ($p < 0.001$) from all other crop rotations except maize-other and other-other. With respect to DON content, the difference was not significant from samples with the rotations cereal-sugar beet, maize-cereal and maize-other (Figure 9). Cultivating maize after maize before barley was however a rare rotation with none of the organic farmers, only 2% of the Extenso and 8% of the PEP farmers. Samples from crop rotations with two years cereals or two year pasture in a row before barley had the lowest average FG incidence and DON content (Figure 9). Since some crop rotation sequences occurred only rarely, combinations with less than five samples (16 samples) were excluded from the statistical analysis, leading to plotted results of 424 samples.

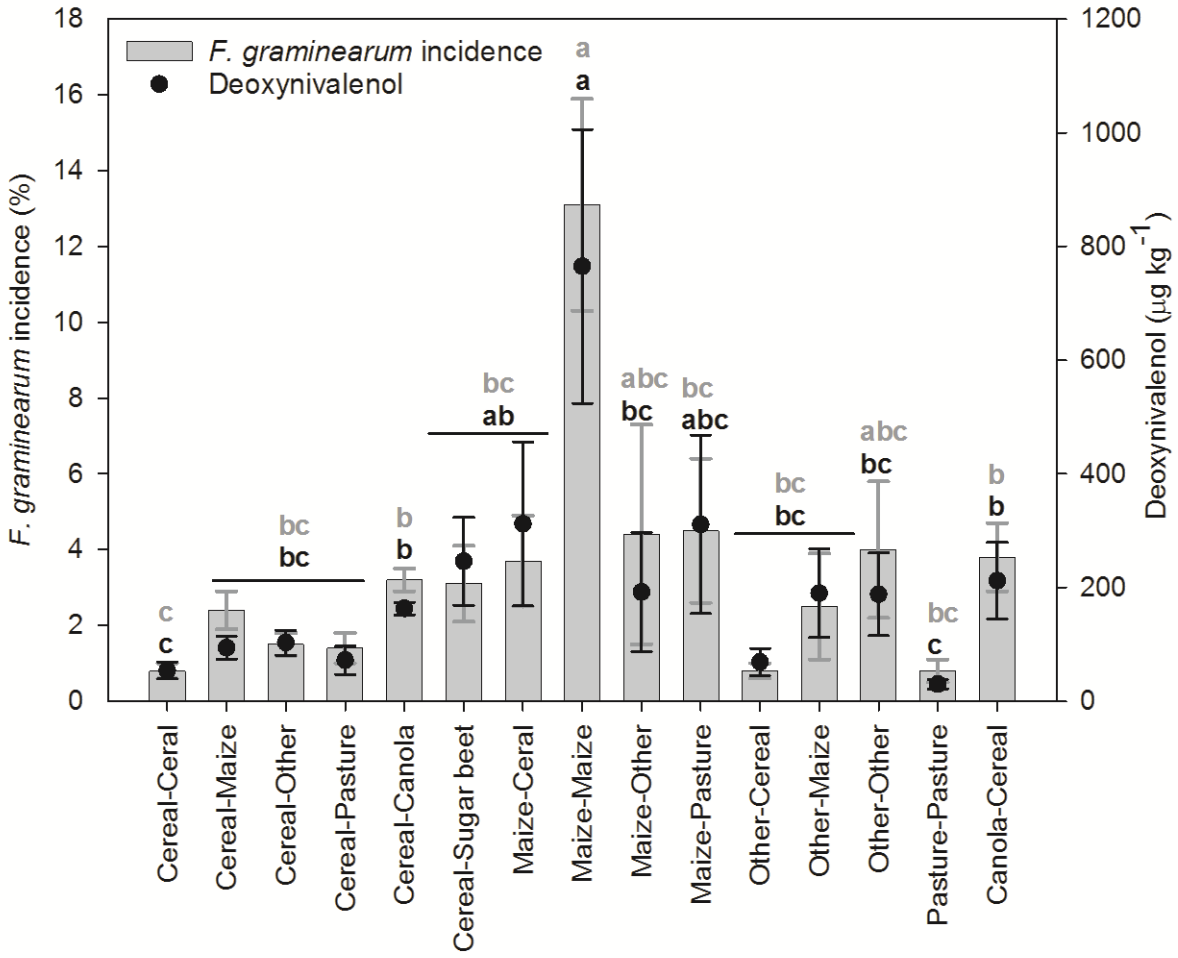

Figure 9: Effect of the previous two crops on *Fusarium graminearum* incidence (%) and deoxynivalenol content (µg kg^{-1}) in Swiss barley samples, collected in 2013 and 2014, n = 424. Error bars represent the standard error of mean, means with the same letters are not significantly different according to a Tukey-Kramer test for interactions at $\alpha = 0.05$. Meanings in cropping factors are as explained in the main text.

Fertilisation, fungicides and growth regulators

Samples with a nitrogen fertilisation of more than 100 kg N ha^{-1} had on average a higher FG incidence and DON content compared with lower nitrogen regimens. The highest DON contamination (318 µg kg-1) was measured in samples with a fertilisation of >200 kg N ha^{-1} (Table 7).

The highest FG incidence (5.6%) and DON content (342.2 µg kg^{-1}) were observed in samples were organic in combination with mineral fertilisers were applied and the lowest (0.4%; 35.9 µg kg^{-1}) if only organic fertilisers were applied (data not shown). Samples without additional nitrogen fertilisation (32 samples), were excluded from the analysis. We suppose that the farmers forgot to add this information in the questionnaire, since a commercial barley production without fertilisation is impractical.

Table 7: Effect of nitrogen fertilisation on *Fusarium graminearum* (FG) incidence and DON content in Swiss barley samples collected in 2013 and 2014. n = number of samples; SEM = standard error of mean. Means with the same letters are not significantly different according to a Games-Howell Test at $\alpha = 0.05$.

Fertilisation [kg N ha^{-1}]	n	FG incidence (%)	DON (µg kg^{-1})
		Mean (SEM)	
1-50	15	0.5 (0.1) c	89 (12) a
51-100	132	1.7 (0.1) bc	96 (14) b
101-150	223	4.0 (0.3) a	224 (24) ab
151-200	29	3.2 (0.3) ab	130 (7) ab
>200	9	3.9 (0.2) abc	318 (21) ab

Application of fungicides and growth regulators is not permitted for Organic and Extenso farmers, while more than 90% of the PEP farmers used both. Fungicide use resulted in a significantly higher FG incidence ($p < 0.001$) and DON content ($p < 0.001$), irrespective of the active ingredient(s). The majority was applied during growth stage 31/32, 37/39 and 49/51 in 2013 and 31/32, 39 and 45 in 2014 (Zadoks et al. 1974). The combination of fungicides belonging to the group of triazoles and strobilurins led to the highest mean FG incidence (6.2%) and DON content (333.8 µg kg^{-1}). The lowest mean incidence (1.4%) and DON content (83.2 µg kg^{-1}) was observed when no fungicide was applied (Table 8). The reduction without application of fungicides ranged from 61% to 77% (FG incidence) and 61% to 75% (DON content) depending on the fungicide.

Table 8: Effect of fungicide group on *Fusarium graminearum* (FG) incidence and DON content in Swiss barley samples collected in 2013 and 2014. n = number of samples; SEM = standard error of mean. Means with the same letters are not significantly different according to a Games-Howell test at $\alpha = 0.05$.

Fungicides	n	FG incidence (%)	DON (µg kg^{-1})
		Mean (SEM)	
Triazoles + strobilurins	71	6.2 (0.5) a	334 (33) a
Strobilurins only	15	3.7 (0.2) ab	189 (12) ab
Triazoles only	126	3.6 (0.2) a	214 (21) a
No fungicide	228	1.4 (0.2) b	83 (11) b

Due to multicollinearity, no statistical analysis was done for the factor growth regulator. The use of growth regulators resulted in a higher average FG incidence (4.6%) compared with no use of growth regulators (1.5%). The same effect was observed for the mean DON contamination (252.8 µg kg^{-1} vs. 88.0 µg kg^{-1}). The reduction was 67% in terms of FG incidence and 65% in terms of DON content.

2.5 Discussion

In the current study, we investigated the FHB causing species complex and the respective mycotoxins in farm samples with natural infection from Switzerland. The non-toxigenic species *Microdochium majus*/*M. nivale* were by far the dominant species isolated from barley, whereas FG and *F. avenaceum* were in both years the dominant toxin producing species.

The high frequency of *Microdochium* species in barley was also observed in other European countries, such as the UK (Nielsen et al. 2014).

In the UK, Nielsen et al. (2014) detected lower amounts of *F. graminearum* DNA in malting barley, but determined *F. avenaceum*, *F. poae*, *F. tricinctum* and *F. langsethiae* (Torp and Nierenberg, no teleomorph known) as dominating species. In a French survey on barley (2000-2002), employing microscopic identification, *F. graminearum* was classified as second or third while *F. poae* or *F. avenaceum* were the predominant species, depending on the year (Ioos et al. 2004).

Hence, the species spectrum in the above mentioned studies are overall in agreement with our findings. However, differences between molecular and microscopic identification and quantification methods exist and may have partially affected the obtained results in terms of the predominant species. Furthermore, environmental and climate conditions have a strong

influence on the *Fusarium* species spectrum (Osborne and Stein 2007) and thus differences between the geographical regions within Europe can be expected.

Deoxynivalenol was the predominant mycotoxin followed by NIV, T-2/HT-2 and ZEA. In accordance with our results, Nathanail et al. (2015a) detected DON in Finnish barley samples most frequently followed by NIV, T-2/HT-2 and ZEA. Similar to our studies, they only found low amounts of T-2/HT-2, which is produced amongst others by *F. langsethiae* (Torp and Nirenberg 2004). The low occurrence of T-2/HT-2 in our samples without identifying *F. langsethiae* could be due to the agar plate method, which might not be suited to recover this species from grains since it grows slowly and might easily be overgrown (Torp and Nirenberg 2004). However, in an extensive study using a great number of *F. langsethiae*, *F. poae* and *F. sporotrichioides* isolates, Thrane et al. (2004) observed that some *F. poae* strains are also able to produce T-2/HT-2, although the majority was produced by *F. langsethiae*.

The higher NIV content in 2014 correlated with the higher occurrence of *F. poae*, which is a known NIV producer (Desjardins 2006). This is in line with Yli-Mattila et al. (2008), who described a correlation between *F. poae* DNA and NIV content in barley and reported that *F. poae* seems to be the most important NIV producer. Although some isolates of *F. graminearum* and *F. culmorum* are known to produce NIV (cited in Desjardins 2006), we did not observe a significant correlation between these species and the NIV content.

Since not all farmers indicated the flowering date, the relation between weather conditions and *Fusarium* species infection and toxin contamination remains unclear. Furthermore, due to the overall low occurrence/contamination, no influence of the weather during calculated flowering periods (based on the sowing date and the geographic origin) at the different sites was detected. Also, most barley varieties are flowering while still in the boot stage and thus the ear is sheltered by the flag leaf which may prevent an infection.

A central aspect of this study was to elucidate the effect of agronomic factors within the different cropping systems. Differences between the varieties have been observed, and overall, winter varieties showed a greater FG incidence and a higher DON content. Only four varieties (Caravan, Cassia, Meridian and Semper) were grown in all three production systems. Generally, these varieties showed a lower FG occurrence and DON content under Organic farming systems. However, since the sample size was rather low, caution has to be taken in interpreting the results. Furthermore, the variety Quench showed the lowest FG incidence and DON content, but was only cultivated by organic farmers. Hence, lower infection rates and smaller amounts of DON could have been due to other factors such as different crop rotation or tillage. In a survey in Norway, Bernhoft et al. (2012) found statistically significant differences between

commonly grown varieties in terms of mycotoxins and *Fusarium* occurrence and observed less occurrence in organically produced cereals. For this reason, we also investigated barley samples from Agroscope variety experiments, since these were all grown under the same agricultural practices. However, no differences were found, probably due to the even lower average incidence of FG (0-6%) and no detection of DON (data not shown). In a five year monitoring (2007-2011) on UK malting barley with different varieties, Nielsen et al. (2014) found only one variety that had a significantly lower *Fusarium* DNA content compared with all other varieties. Thus, further studies are needed to elucidate the susceptibility of different barley varieties.

The reason for higher FG incidence and DON contamination in winter varieties compared with spring varieties remains unclear. Although it seems possible that the longer cultivation period of winter varieties provides more time for fungal development and toxin production, we found no positive correlation between vegetation duration and FG incidence or DON content. Likewise, Xue et al. (2004) stated no significant effect of harvesting date on DON concentration in wheat, but observed a higher FG incidence in delayed harvest samples, which could be explained by more colonisation time and a wider infection window.

The current study has demonstrated that maize as a previous crop contributes to a higher FG infection and DON content. This finding is not unexpected since FG can infect maize and survive saprophytically on these residues and thus can serve as an inoculum in the next growing season (Osborne and Stein 2007). Similar results were obtained in other field studies on FHB in wheat and barley (Dill-Macky and Jones 2000). The cultivation of 2-years maize before barley resulted in the highest FG incidence and DON content. We assume that this led to an increased disease pressure and favoured the infection conditions. *Fusarium graminearum* can also survive on canola residues (Fernandez 2007), and hence it was not surprising that in samples from fields with previous crop canola, a higher FG incidence and DON contamination was detected compared with other previous crops.

Ploughing of residues from the previous crops revealed a lower occurrence of FG and DON. Ploughing buries potentially infected crop material and the decaying by microorganisms is favoured (Pereyra and Dill-Macky 2008). The greatest effect of reduced tillage was observed in combination with the previous crops cereals and maize, which are both hosts for FHB causing species, as shown in barley by Fernandez et al. (2007).

The reduction of FG incidence and DON content through additional chopping of residues from the previous crop maize was also observed in a 5-year field experiment by Vogelgsang et al. (2011). The treatments with a field shredder and other mulching equipment reduced disease symptoms, disease incidence and DON content in wheat. Most probably, the decomposition

was accelerated, leading to reduced survival of the fungal inoculum. However, in a microcosm study by Vestergaard et al. (2001), no effect on decomposition was observed when maize leaves were finely ground. We hypothesise that the resulting surface enlargement could also lead to space and nutrition competition between FG and other microorganisms and thus reduce the inoculum potential. Moreover, we assume that earthworms can use chopped residues more efficiently and thus increase the reduction of crop debris. This effect was shown in field studies by Wolfarth et al. (2011) with wheat straw. Here, earthworms reduced the amount of *Fusarium* infected straw, which was a more attractive food compared with the control.

As the application of fungicides during anthesis in barley is currently not permitted in Switzerland, we assume that fungicides were not used to control *Fusarium* species. The use of fungicides before anthesis may have reduced or eliminated the incidence of other fungi on the plant surface. As a result, the potential for the colonisation on the plants or crop debris could have been higher and may have resulted in an easier spreading and infection which in consequence led to a higher FG incidence and DON content.

Triazole fungicides are known to be effective against FG and *F. culmorum*, whereas strobilurins are more effective against *M. majus*/*M. nivale* in wheat, as reported by Simpson et al. (2001). They observed a lower occurrence of FG when triazoles were applied and a lower occurrence of *Microdochium* species when strobilurins were applied. In the last years, however, resistance of *Microdochium* species against strobilurins was observed in barley (Nielsen et al. 2013).

The majority of fungicide studies were carried out with wheat and observed a reduction of FHB and mostly of DON after the application of strobilurins, triazoles or a mixture of both (Blandino et al. 2006; Haidukowski et al. 2012). A direct comparison with these results is difficult to achieve, since in our survey, not all farmers indicated the time of fungicide application and the anthesis period.

A higher FG incidence and DON content was detected when growth regulators were applied. The ears of shortened plants are closer to the ground and thus an infection through ejected ascospores is favoured (Maji and Imolehin 2002). The modified crop characteristic was described by Bernhoft et al. (2012), who assumed that plants become bushier, which enhances the humidity and the spreading of *Fusarium* species. They also stated that higher nitrogen fertilisation favours lodging due to heavier ears, which is known to increase infections. Additionally, depending on the time of application and the dose, the plant is set under abnormal stress, which makes it in general more susceptible to plant diseases.

With increasing nitrogen fertilisation (> 100 kg ha^{-1}), FG incidence and DON content was up to twice as high compared with lower doses, irrespective if it was applied as organic or chemical

fertiliser. Lemmens et al. (2004b) observed an increase of visual disease symptoms and DON content with increasing nitrogen rate in wheat and hold the changed microclimate or longer flowering period responsible for that finding. In contrast, nitrogen deficiency may render plants vulnerable to disease and in fact, Yang et al. (2010) concluded that FG infection and mycotoxin levels were more severe in barley plants with low nitrogen fertilisation. Our results indicate that following the current fertilisation recommendations for barley in Switzerland (110 kg N ha^{-1} for winter barley and 90 kg N ha^{-1} for spring barley), the risk of a FHB infection can be reduced. However, the data must be interpreted with caution, since the organic fertilisation was calculated using averaged values and data about N_{min} were not inquired. Furthermore, we observed that the organic farmers fertilised less N compared with Extenso and PEP farmers and thus other factors (tillage, previous crop) could have a bigger influence on FG incidence and DON content.

2.6 Conclusions and Outlook

Our two year barley monitoring clearly demonstrated that the occurrence of *Fusarium* species and their respective mycotoxins is affected by several cropping factors. Frequently, not only one but rather the combination of different factors have to be considered, since maize as the previous crop together with reduced tillage led to the highest infections and DON contents. Thus, the entire cropping system should be taken into account to retrieve factors influencing the respective *Fusarium* species. The fact that we observed a lower occurrence and contamination in organically cultivated barley compared with samples from Extenso or PEP was probably due to cropping techniques that reduce the inoculum potential, such as a wider crop rotation, ploughing, no use of fungicides or growth regulators and less nitrogen fertilisation.

Since the overall disease pressure in 2013 and 2014 was low, further monitoring of barley is imperative to confirm our findings or to observe potential changes in the *Fusarium* species spectrum and their respective mycotoxins. Studies that investigate the differences between the flowering of barley and wheat in terms of infection and epidemiology should be conducted to increase knowledge about the infection process. Results from the current study together with further monitoring data can be used to develop cropping systems and disseminate recommendations that allow farmers to produce cereals with low risks of mycotoxin contamination.

2.7 Acknowledgements

This work was funded by the Swiss National Research Programme 69 “Healthy Nutrition and Sustainable Food Production” [grant number 406940_145210]. The authors thank the Swiss cantonal plant protection officers who providing addresses of barley growers; and all participating growers for kindly providing samples of their harvest and for completing the questionnaires. Excellent technical support was provided by Irene Bänziger, Eveline Jenny and Andreas Kägi.

No conflict of interest was reported by the authors.

2.8 Supplementary Material

Table 9: Questionnaire (including sampling instructions) about agronomic data; ÖLN ("Ökologischer LeistungsNachweis" = proof of ecological performance (PEP)) is the minimum standard for an environmentally friendly agriculture to obtain direct payments; Extenso renounces additionally the use of growth regulators, fungicides, insecticides and synthetic stimulators of natural defence mechanisms; Organic totally prohibits the use of synthetic plant protection products and mineral fertilisers.

Name and address Tel.: E-Mail:			ÖLN ☐ Extenso ☐ Organic ☐
Variety:	Previous crop, if maize, please indicate if silage or grain maize:	Pre-Previous crop:	
Did you observe Fusarium head blight in your field? Yes ☐ No ☐ comment:			
Combine with crop residue chopper ☐ yes ☐ no ☐ unknown			
Additional chopping of crop residues ☐ yes ☐ no			
Plough ☐ yes ☐ no			
Incorporation of crop residues: chisel plow ☐; disc harrow ☐; Other: ☐ spring tine harrow ☐; rotary harrow ☐; rototiller ☐ ……			
No-till ☐ yes ☐ no			
Sowing date:	Begin of flowering (DC 61):	Harvesting date:	
N-fertilisation: kg N / ha	1	2	3
Fungicide, if used	Product …… …… ……	Date …… …… ……	Growth stage …… …….. ……
Growth regulator, if used	Product	Date	Growth stage
Instructions for sub sampling The grain sample can be taken directly after threshing form the combine, from trailers or from the silo. Please take ten individual subsamples and mix them carefully to one sample of approximately 1kg for the respective field. For transportation of the samples, please fill in the sample in the plastic bag and afterwards in the paper bag (protection against damage). Fold the paper bag two times and lock it with the clamp.			

3 Occurrence of Fusarium species and mycotoxins in Swiss oats – Impact of cropping factors

published in European Journal of Agronomy

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3.1 Abstract

Between 2013 and 2015, 325 samples of commercially grown oats were collected in Switzerland along with data on respective cropping factors. The incidence of different *Fusarium* species was determined using a seed health test and quantitative PCR was used to measure the amount of *F. poae* and *F. langsethiae* DNA. Mycotoxins were quantified by LC-MS/MS. Among all *Fusarium* species, *F. poae* was found to be dominant whereas T-2/HT-2 toxins were the major mycotoxins. Samples from fields with the previous crop cereal showed the highest concentrations of T-2/HT-2. Higher amounts of nivalenol (NIV) and T-2/HT-2 were detected in samples from fields with reduced tillage compared with samples from ploughed fields. Furthermore, we observed a higher contamination with NIV and T-2/HT-2 in winter sown varieties compared with spring sown varieties. Results from the current study are highly valuable to develop recommendations for optimised cropping systems that reduce the risk of mycotoxin contamination of oat grains.

3.2 Introduction

One of the most devastating cereal diseases, Fusarium head blight (FHB), is caused by a range of *Fusarium* species (Parry et al. 1995). Infections do not only result in yield reduction and thus economic losses (Nganje et al. 2004), but also in the production of mycotoxins, particular trichothecenes or the mycoestrogen zearalenone, which threaten human and animal health (Desjardins 2006). Thus, the European Commission established maximum limits for different mycotoxins in 2006 and indicative levels for the sum of T-2 toxin and HT-2 toxin (T-2/HT-2) in 2013 (The European Commission 2006; The European Commission 2013). Recently, the European Food Safety Authority (EFSA) also established a tolerable daily intake (TDI) for T-2/HT-2 of 0.02 µg kg^{-1} body weight and an acute reference dose of 0.3 µg kg^{-1} body weight (EFSA 2017).

In Switzerland, *F. graminearum* (FG) and its mainly produced mycotoxin deoxynivalenol (DON) is the most dominant species, and mycotoxin, respectively, both in barley (*Hordeum vulgare* L.) and in wheat (*Triticum aestivum* L.) (refer to chapter 2; Vogelgsang et al. 2009). Less is known about the occurrence of *Fusarium* species and mycotoxins in Swiss oats (*Avena sativa* L.). Another commonly occurring *Fusarium* species in cereals is *F. poae* (FP), which produces amongst others the mycotoxin nivalenol (NIV) (Desjardins 2006).

Fusarium langsethiae (FL) is thought to be the primary producer of T-2/HT-2 in northern Europe and the UK, whilst the other T-2/HT-2 producer, *F. sporotrichioides*, occurs only occasionally in these areas (Imathiu et al. 2013b; Fredlund et al. 2013; Hofgaard et al. 2016a). The same holds true for the occurrence of *F. sporotrichioides* in Switzerland, where it was not found neither in barley (refer to chapter 2) nor in wheat, and only occasionally in maize (Dorn et al. 2009; Vogelgsang et al. 2009).

On wheat and barley, severe FHB symptoms on cereal heads or grains are frequently a sign of FG infection and DON contamination. However, infection with FP or FL usually does not lead to typical FHB symptoms (Imathiu et al. 2009; Stenglein 2009). Thus, healthy looking grains can contain elevated amounts of mycotoxins like T-2 and HT-2. These toxins are more often detected in oats than in barley or wheat (Hofgaard et al. 2016a; Edwards et al. 2009). In addition, mycotoxins as T-2/HT-2, NIV and DON are known to decrease feed intake. Because T-2/HT-2 and NIV show a higher toxicity compared with DON (Placinta et al. 1999; Nielsen et al. 2009; D'Mello et al. 1999), even lower concentrations of T-2/HT-2 or NIV can have the same or even a worse effect on animal health compared with higher concentrations of DON in contaminated cereals. T-2 is thought to be mainly responsible for the death of more than 100.000 people in

the former Soviet Union between 1942 and 1948 after consumption of mouldy grains (cited in Ramos et al. 2011).

Nevertheless, oats is a highly valuable cereal because it contains elevated levels of dietary fibre such as β-glucan, which is amongst others known to moderate postprandial blood glucose and insulin peaks as well as to lower the cholesterol level (Brennan and Cleary 2005; Wang and Ellis 2014). Furthermore, oats has a high protein content and a favourable lipid profile (Welch 1995). Oat flour is often used in baby food because of its nutritional profile and other useful characteristics such as stabilisation due to its high water-holding capacity (Welch 1995). However, for safe consumption, especially as a baby food, and for use as a beneficial ingredient, oats must be free of mycotoxins (Lombaert et al. 2003).

In Switzerland, the application of fungicides against *Fusarium* in oats is not permitted (Hochstrasser 2016). Thus, preventative cultivation measures, such as variety, tillage or crop rotation, have to be chosen to reduce the risk of mycotoxin contamination in oats. Therefore, knowledge about the prevailing *Fusarium* species and mycotoxins, and possible influencing factors, is fundamental for preventing contaminated grains entering the supply chain. The aims of the present study were (1) to identify the dominant *Fusarium* species and mycotoxins in Swiss oats and (2) to determine potential agronomic factors associated with the infection and mycotoxin contamination.

3.3 Material and Methods

Collection and processing of samples

Collection of oat samples, was done by addressing several hundred farmers to send minimum one kg of their oats harvest, along with the respective agronomic data to the Agroscope research station in Zurich-Reckenholz as described for barley in chapter 2. In 2013, 2014 and 2015, 92 samples from 11 cantons, 66 samples from 13 cantons and 167 samples from 13 cantons, respectively, were obtained. A detailed array of the number of samples per canton can be seen in Table 13 in the supplementary Material (chapter 3.8). The samples arrived with an average humidity of 11%, and were stored at 5 °C until further processing. From all samples, the hectolitre weight was measured using a grain analysis computer 2100 (Dicky-John Corporation, Auburn, Illinois, USA). Representative subsamples of each sample were obtained by using a riffle divider (Schiertz & Hauenstein AG, Arlesheim, Switzerland). A subsample of six gram hulled oats was taken to determine the *Fusarium* species incidence. Subsamples of 150 g hulled

oat grains were ground with a centrifugal mill (Retsch® ZM 200, Schieritz & Hauenstein AG, Arlesheim, Switzerland) using a 0.25 mm screen for mycotoxin and DNA analysis. The flour samples were stored at -20 °C until analyses.

Identification of *Fusarium* species

The seed health test on potato dextrose agar (Oxoid, Basingstoke, UK) as described in Vogelgsang et al. (2008b) was conducted with 100 grains per sample and expressed in percentage infested grains. The morphological identification of *Fusarium* species followed the manual of Leslie and Summerell (2006).

Extraction of fungal DNA

For DNA extraction, oat flour of 50 ± 2 mg (dry weight) were transferred into 1.2 ml polypropylene cluster tubes (96 well propylene cluster tubes, Corning, USA) and locked with cap strips for storage at -20 °C until further analysis.

DNA extraction was done with the NucleoSpin® 96 Plant II Kit (Macherey-Nagel, Düren, Germany) with the aid of one autoclaved tungsten bead per tube. Previous DNA extractions from oats resulted in low yields of total DNA, so the manufacturer's protocol was altered at the cell lysis step by adding 600 µl lysis buffer PL1 (Macherey-Nagel, Düren, Germany) and 12 µl RNAse (Macherey-Nagel, Düren, Germany) and then shaking all samples twice for 30 sec/30 Hz per run in a TissueLyser II (Qiagen®, Hombrechtikon, Switzerland). Subsequently, the samples were incubated at 65 °C for 30 min. After the washing procedure, all binding columns were centrifuged for five minutes at 5'800 rpm, sealed with a gas permeable foil and dried in an incubator (15 min, 37 °C). After the elution step, all samples were stored at -20 °C until further analysis.

The amount of total DNA in the samples was determined by measuring the emission of the fluorescent PicoGreen® reagent (Promega, Madison, USA) bound to double stranded DNA based on the emitted fluorescence of a known concentration of a serially diluted DNA standard (HeringSperm DNA, Promega, Madison, USA) in a range of 502 nm to 523 nm after excitation at 480 nm in a Cary Eclipse UV/Visible Spectrophotometer (Varian, Walnut Creek, USA). Prior to measuring the samples, they were diluted 1:20 with Tris(hydroxymethyl)-aminomethan ethylenediaminetetraacetate (Tris EDTA) buffer and incubated with PicoGreen reagent. The dilution series of the DNA standard ranged from concentrations of 1 to 20 ng/µl.

Quantification of *Fusarium* species DNA

Quantitative PCR (qPCR) was performed to determine the amount of FL and FP DNA in milled grain samples. All reactions were analysed in a CFX96™ Real-Time PCR Detection System–IVD (Bio-Rad) in a 96-well plate format (Hard-shell full-height 96-well semi-skirted PCR plates, BioRad, Hercules, USA). In every performed assay, all standards and the negative control (double distilled water) were run as triplicates.

The PCR protocol specifications and thermocycling parameters for the FL DNA quantification were obtained from Edwards et al. (2012) and adapted to the available reaction mixes and laboratory devices. The amplification mix consisted of the primer pairs (Microsynth AG, Balgach, Switzerland) FlangF3 (5'-CAAAGTTCAGGGCGAAAACT) and LanspoR1 (5' TACAAGAAGACGTGGCGATAT) (Wilson et al. 2004) (1 µM) and IQ SYBR® Green Supermix (Bio-Rad, Cressier, Switzerland). The initial melting curve analysis of Edwards et al. (2012) was changed from 95 °C/ 15 sec to 95 °C / 10 sec for heating, followed cooling down to 65 °C instead of 55 °C preceded, by a graduate rise to 95 °C at a rate of 0.05 °C/sec. The plasmid, needed for establishing the standard curve, was prepared according to instructions of the Promega pGem®-T and pGem®–T Easy Vector Systems Kit (Promega, Madison, USA) with *E. coli* DH5α. Plasmid DNA from *E. coli* DH5α recombinants was extracted and purified with the QIAprep® Miniprep Kit (Qiagen Scientific, Venlo, Netherlands).

The amplification mix used for the FP qPCR consisted of primer pairs
ACL1-F160 (5'-CCATCCCCAAGACACTGAG) and
ACL1-R330 (5'-TACAAGTTGCTRCAAGCCC) and
TaqMan-Probe (ACL1_poae1_probe; 5'-GTTCTTCTCAGGACTTTACCCCGAAAGCC) (Qiagen AG, Hombrechtikon, Switzerland), which was used according to manufacturer's instructions. The PCR programme had an initial denaturation for 10 min at 95 °C followed by 40 cycles with 30 sec at 95 °C (denaturation) and 60 sec at 62 °C (annealing/extension) followed by fluorescence measurement.

After performing several qPCR runs, the limit of determination (LOD) and the limit of quantification (LOQ) were determined from the dilution series of the standard (plasmid DNA template) for both *Fusarium* species. Therefore, the double amount of the lowest standard (SQ 20) was set as LOQ and the LOD was set as one tenth of the LOQ. Sample values below the LOD or LOQ were replaced by a constant value of LOD/2 or LOQ/2. The number of copies per ng DNA extracted (total DNA) was determined according to the size of the used plasmid. Therefore, the number of base pairs was divided by the size of the base pairs of the plasmid.

Mycotoxin analysis

LC-MS/MS was performed as described in chapter 2 but using 0.3 mg Alox:Celite (50:50, Alox activated 6 h by 400 °C) instead of 0.15 mg for extract cleaning. The method was validated by recovery experiments of differently spiked samples (200 and 2500 µg kg^{-1}). The recovery range for the measured trichothecenes, NIV, DON, fusarenon-X (FUS-X), acetyldeoxynivalenol (AcDON), neosolaniol (NEO), diacetoxyscirpenol (DAS), T-2, HT-2 and zearalenone (ZEA), was between 85% and 130%. The limit of quantification (LOQ) was determined as ten times the baseline noise. The limit of detection (LOD) was determined as three times the baseline noise. The ranges of LOQ and LOD for DON, NIV, T-2 and HT-2 are presented in Table 11. The LOQ and LOD vary because the samples were measured in different runs. Mycotoxin concentrations below the respective LOQ or LOD were calculated as LOQ/2 or LOD/2, respectively.

Statistical analysis

Statistical analysis and assembly of figures was conducted with 'R' version 3.3.1 (R Core Team 2015) and 'R Studio' version 0.99.903 (R Studio Team 2015). The packages 'MuMIn' (Barton 2016) and 'nlme' (Pinheiro et al. 2016) were used for model selection.

Homogeneity of variance and normality of residuals were checked graphically using plots of fitted values versus the root of the standardised residuals and normal Q-Q plot, respectively. To meet homogeneity of variance and normal distribution of residuals, qPCR data and mycotoxin data were log and seed health test data were arcsine (square root) transformed, respectively.

To determine influencing factors on FP and FL DNA, NIV and T-2/HT-2 concentrations, a multiple linear regression model was employed using stepwise backward/forward regression. The obtained regression models were evaluated by using the Akaike Information Criterion (AIC). All factors were checked for multi-collinearity using a correlation matrix, factors with a correlation coefficient > 0.3 were removed from the model. The model with the greatest explanation rate according to the AIC value was used for further analysis. The used model included five management variables: tillage, previous crop, pre-previous crop, additional chopping of crop residues and growth regulator as well as the combination of the factors previous crop + tillage, previous crop + pre-previous crop and previous crop + additional chopping as explanatory variables on the dependent variables FP DNA, FL DNA, NIV and T-2/HT-2. The predictive performance of the model, as well as the F-value and the significance

probability value associated with the F-value for the model used with data of the respective year or with combined data of all three years are shown in Table 14 to Table 17. An analysis of variance was then used to find significant influencing factors.

The significant influencing factors of tillage, previous crop and growth regulator were further analysed with the Games-Howell post-hoc test for uneven sample number with the package 'userfriendlyscience' (Peters 2016). Because this test is not applicable for interactions, the Tukey-Kramer post-hoc test was performed with the aid of the package 'multcomp' (Hothorn et al. 2008) to investigate the interactions between the factors previous crop x additional chopping, previous crop x tillage and previous crop x pre-previous crop. Data analysis was performed with a probability value of 5% ($\alpha = 0.05$). For figures and tables, untransformed data were used. Pearson's correlation coefficient (r) between mycotoxins, qPCR data and seed health test data was calculated on transformed data (see above) using the package 'agricolae' (de Mendiburu 2015).

3.4 Results

Fusarium species spectrum in Swiss oat samples from 2013 to 2015

Overall, nine different *Fusarium* species were detected in Swiss oat samples and 97% of the samples were infected with at least one species. Throughout all three years of the monitoring, these were in descending order of incidence, FP, FL, *F. avenaceum*; (FA), FG, *F. culmorum*, *F. equiseti*, *F. crookwellense* and *F. tricinctum*. *Fusarium poae* was the predominant species in all three years (in 2013, 2014 and 2015, 55%, 57% and 87% of all isolated *Fusarium* species, respectively). The second most occurring species in 2013 was FG (17%), in 2014 FA (28%) and in 2015 FL (11%). In 2013, FL was the third most occurring species with 16%, in 2014, FG (11%) and in 2015, FA (1%). All other *Fusarium* species occurred in each year with one percent or below (Figure 10). In 2014, we observed only 1% FL, and FG was below 1% in 2015. The non-toxigenic species *Microdochium nivale*/*M. majus* were the most occurring species in 2014 (41%), but second most in 2013 (28%) and third most in 2015 (4%). However, because these species do not produce mycotoxins, they were not used for plotting of species spectra or statistical analyses.

In 2013, 2014 and 2015, the highest FP incidences were 18%, 22% and 58%, respectively. For FL, the highest incidences in 2013, 2014 and 2015 were 11%, 1% and 15%, respectively. The mean incidence for FP were different in all three years (ranging from 3.9 to 10.0%), whereas

the mean incidence for FL remained more stable (0.1 to 1.3%) (Table 10). The fungal amount in oat grains ranged from 6.9 to 44.5 copies ng total DNA^{-1} for FP and 5.0 to 14.5 copies ng total DNA^{-1} for FL. The highest FP amount in 2013, 2014 and 2015 were 74 copies ng total DNA^{-1}, 345 copies ng total DNA^{-1} and 80 copies ng total DNA^{-1}, respectively. The highest FL amount in 2013, 2014 and 2015 were 177 copies ng total DNA^{-1}, 389 copies ng total DNA^{-1} and 59 copies ng total DNA^{-1}, respectively.

The comparison of only FP and FL revealed a higher incidence of FL in the qPCR data compared with the seed health test data. We observed 37%, 24% and 22% more FL in the years 2013, 2014 and 2015, respectively. The correlation of the qPCR and the seed health test data was stronger for FP ($r= 0.47$, $p<0.001$) than for FL ($r=0.39$, $p<0.001$).

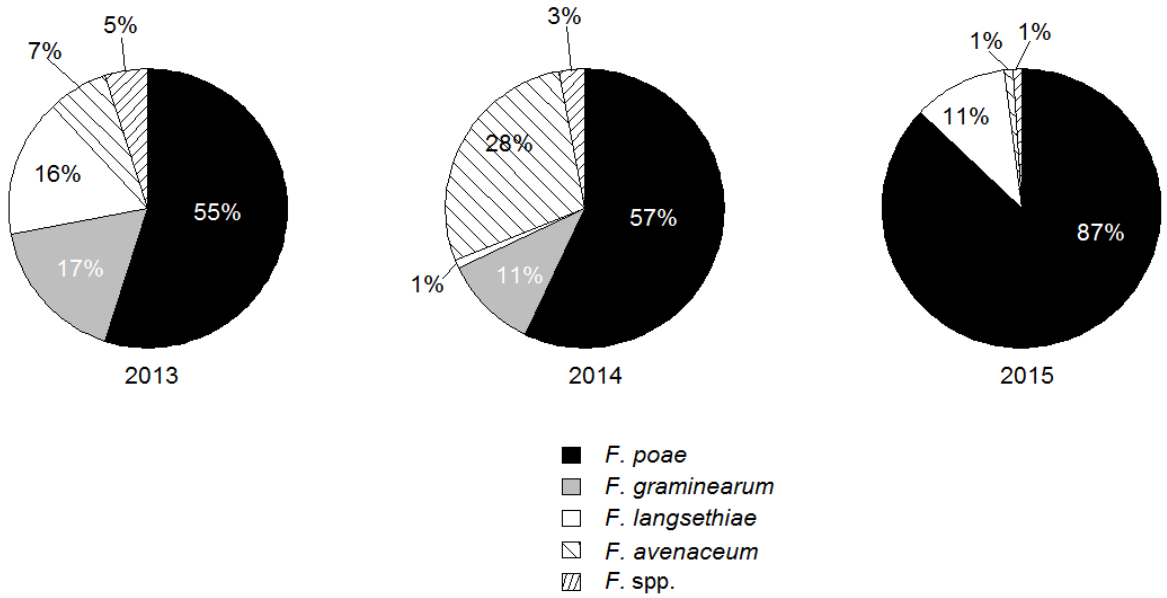

Figure 10: Ratio of different species from all detected *Fusarium* species in Swiss oats samples collected in 2013, 2014 and 2015; *F.* spp.: *Fusarium* species with an incidence of < 1%; number of samples: 2013 = 92; 2014 = 66; 2015 = 167.

Table 10: Incidences of *Fusarium poae* (FP) and *F. langsethiae* (FL) according to seed health tests and mean copies/ng total DNA of FP and FL according to quantitative PCR in Swiss oats samples collected between 2013 and 2015, described by mean value and standard error of the mean. Other *Fusarium* species with lower incidences are not indicated. n = number of samples.

Year	n	Mean incidence of *Fusarium* species (%) ± standard error		Mean copies/ng total DNA of *Fusarium* species ± standard error	
		FP	FL	FP	FL
2013	92	3.9 ± 0.8	1.0 ± 0.4	6.9 ± 1.1	9.2 ± 2.3
2014	66	5.4 ± 1.2	0.1 ± 0.1	44.5 ± 7.0	14.5 ± 6.1
2015	167	10.0 ± 1.5	1.3 ± 0.4	10.3 ± 1.1	5.0 ± 0.7

Fusarium mycotoxins in Swiss oat samples from 2013 to 2015

In 2013, 2014 and 2015 in 52 samples (56%), 62 samples (94%) and 147 samples (88%) at least one mycotoxin above the LOD was measured. The mycotoxin analysis with LC-MS/MS revealed T-2/HT-2 to be the predominant mycotoxins in 2013, 2014 and 2015 with average concentrations of 214 µg kg^{-1}, 178 µg kg^{-1} and 295 µg kg^{-1}, respectively. The second most occurring mycotoxin in 2014 and 2015 was NIV, whilst DON was second in 2013 (Table 11). The average contamination with T-2/HT-2 and NIV was highest in 2015, whereas the average contamination with DON was highest in 2013. Zearalenone contamination was generally low in all three years with 1% of the samples above LOD in 2013 and 2015 and 32% in 2014. The highest average concentration (20.7 µg kg^{-1}) and the maximum detected value (306 µg kg^{-1}) were measured in 2014. The average contamination was 7.5 µg kg^{-1} in 2013 and 2.1 µg kg^{-1} in 2015.

The European Commission guideline value for unprocessed oats with husk for human consumption (The European Commission 2013) of 1'000 µg kg^{-1} T-2/HT-2 was exceeded in 2013 in five samples (5%), 2014 in 1 sample (2%) and 2015 in 11 samples (7%). The maximum limit for unprocessed oats for DON (1'750 µg kg^{-1}) was not exceeded in any of the years, the maximum limit for unprocessed oats for ZEA (100 µg kg^{-1}) was only exceeded in 3 samples from the year 2014 (5%).

Concentrations of NEO above the LOD (50 µg kg^{-1} in 2013 and 118 µg kg^{-1} in 2015) were found in samples with a high contamination of T-2/HT-2 (631 µg kg^{-1} in 2013 and 4'880 µg kg^{-1} in 2015). Nevertheless, NEO was detected in 9% of the samples in 2013 and 2015 and in 23% of the samples in 2014 but only low levels (9-118 µg kg^{-1}) were measured (data not shown). Still, we observed a strong correlation ($r=0.69$, $p<0.001$) between T-2/HT-2 and NEO. No correlations were observed between DON and NIV (r=0.009), DON and T-2/HT-2 (r=-0.15) or

NIV and T-2/HT-2 (r=0.05). This verifies our observation that the amounts of DON and NIV were lower when high levels of T-2/HT-2 were present and less NIV was detected whit higher levels of DON.

Mycotoxin concentrations were significantly positively correlated with both fungal incidence and qPCR data. For FP incidence and NIV, the correlation was slightly stronger (r=0.50, p<0.001) than for NIV and qPCR data (r=0.45, p<0.001). The opposite was observed for FL and T-2/HT-2, where the incidences showed a weaker correlation (r=0.52, p<0.001) compared with the qPCR data (r=0.66, p<0.001). The strongest correlation (r=0.80, p<0.001) was detected between the two mycotoxins T-2 and HT-2.

Co-occurrence of mycotoxins was observed in several samples: in 159 samples, only one mycotoxin was detected, whereas two different mycotoxins were detected in 88 and three in 37 samples. Four different mycotoxins were detected in 12 samples, five in four samples and six in six samples. In 22 samples, none of the measured mycotoxins were found. For these calculations, T-2 and HT-2 are displayed together as T-2/HT-2, because they always occurred together. The second most co-occurring mycotoxins were NIV and T-2/HT-2 (169 samples), NIV and DON (120 samples) and DON and T-2/HT-2 (119 samples).

Table 11: Concentrations of deoxynivalenol (DON), nivalenol (NIV) and T-2/HT-2 toxins in Swiss oats samples collected between 2013 and 2015 described by mean value, standard error of the mean, maximum detected value and percentage of samples with mycotoxins above the limit of detection (LOD). n = number of samples.

Year	n	Mean mycotoxin concentration (µg kg^{-1}) ± standard error of the mean (maximum detected value) percentage of samples above LOD				
		DON	NIV	HT-2	T-2	T-2/HT-2
2013	92	85 ± 39 (1328) 44	66 ± 6 (211) 5	181 ± 64 (1715) 63	60 ± 18 (453) 65	241 ± 80 (2168)
2014	66	48 ± 22 (405) 45	96 ± 24 (435) 98	141 ± 48 (962) 73	37 ± 12 (237) 76	178 ± 57 (1115)
2015	167	38 ± 12 (735) 58	164± 37 (1653) 90	226 ± 77 (3789) 46	69 ± 20 (1091) 74	295 ± 96 (4880)

Limits of quantification (LOQs) (µg kg^{-1}): DON = 10-60, NIV = 20-210, T-2 = 20-30, HT-2 = 30-80
LODs (µg kg^{-1}): DON = 4-20, NIV = 10-50, T-2 = 1-10, HT-2 = 1-20.
Mycotoxin concentrations below the respective LOQ or LOD were calculated as LOQ/2 or LOD/2, respectively.

Association between crop rotation and tillage on fungal infection and mycotoxin contamination of grains

The analysis on the association between cropping factors on fungal infection and mycotoxin contamination was performed for the two most dominant species, (FP) and (FL), and their respective mycotoxins NIV and T-2/HT-2. Data were pooled over the years if the influencing cropping factors had the same effect in all three years. Within the different cropping factors, groups were assembled to obtain meaningful sample sizes (n>10). Because of the discrepancy between the observed incidences of FP and FL in the seed health tests and the analysed mycotoxins, we decided to analyse all samples on their content of FP and FL DNA. Only the qPCR data were used for the following analysis on influencing cropping factors. The grouping of previous crops and the crop before the previous crop in the further text referred as pre-previous crops was done as follows: previous crops were cereal (n=201); (emmer (n=2), rye (n=2), spelt (n=4), oats (n=7), triticale (n=21), barley (n=44), wheat (n=121)), maize (n=70), pasture (n=31) and 'Other' (n=23) (ten different crops with each less than eight samples); pre-previous crops were cereal (emmer (n=1), spelt (n=2), oats (n=4), rye (n=7), triticale (n=11), barley (n=17), wheat (n=90)), maize, pasture, canola and other (eight different crops, each with less than ten samples).

In all three years, samples from fields with the previous crop cereal had the highest values of T-2/HT-2 (yearly mean values between 259 and 409 μg kg^{-1}) and FL DNA (Figure 11). In contrast, samples from fields with the previous crop pasture showed the lowest T-2/HT-2 concentrations (yearly mean values between 24 and 44 μg kg^{-1}). The differences in T-2/HT-2 content between the previous crop cereal and pasture were significant in all three years (2013: p=0.008, 2014: p=0.007, 2015: p<0.001) (Figure 11). Likewise, the number of FL-DNA copies was lower in samples from fields with previous crop pasture; however, this difference in comparison with the previous crop cereal was significant only in 2015 (p=0.04) (Figure 11) and with the combined data between 2013 and 2015 (p<0.001). A significantly higher T-2/HT-2 content was noticed in samples with the previous crop cereal compared with samples with the previous crop maize in 2014 (p=0.004), 2015 (p<0.001) (Figure 11) and with combined data (2013-2015, p<0.001). The same holds true for the comparison between previous crop cereal and the group of 'Other' previous crops in 2014 (p=0.045), in 2015 (p=0.003) and 2013-2015 (p<0.001). A significant difference between the other previous crops was only found comparing maize and pasture in 2015 (p=0.025) and when all three years were analysed together (p=0.001). The effect of the previous crop cereal resulting in higher T-2/HT-2 contents and FL DNA was further increased if the pre-previous crop was also a cereal (average values of 425 μg kg^{-1};

12 copies/ng DNA extracted, respectively), maize (366 µg kg^{-1}; 19 copies/ng DNA extracted) or 'Other' (444 µg kg^{-1}; 7 copies/ng DNA extracted). The lowest T-2/HT-2 content and amount of FL DNA was measured in samples with two years pasture in a row (28 µg kg^{-1}; 2 copies/ng DNA extracted), which was significantly different ($p<0.001$) from all combinations with previous crop cereal except for cereal-pasture (Figure 12). Because some crop rotation sequences occurred only rarely, combinations with fewer than five samples were excluded from the statistical analysis, leading to plotted results of 308 samples. Neither the previous crop nor the crop rotation of the last two years showed an effect on the FP DNA and the NIV content (data not shown).

A difference ($p=0.045$) in the T-2/HT-2 content between samples from ploughed (n=262) and reduced tillage (n=63; including 7 samples from direct sowing) fields irrespective of the previous crop was only found with combined data from all three years. In those cases, samples from ploughed fields showed a lower content of T-2/HT-2 (240 µg kg^{-1}) compared with the reduced tillage plots (323 µg kg^{-1}) (Table 12). Despite the significant correlation between FL and T-2/HT-2, this effect was not observed for FL DNA.

The combination of previous crop and tillage showed no significant differences in terms of T-2/HT-2 content within the same previous crop. Nevertheless, we observed a lower average T-2/HT-2 content and FL DNA in samples from fields with previous crop cereal or maize in combination with plough (Table 12).

For NIV, we observed in 2013 a significantly ($p=0.017$) higher content (84 µg kg^{-1}) in samples from reduced tillage fields compared with ploughed fields (60 µg kg^{-1}), irrespective of the previous crop. For the years 2014 and 2015, this effect was also observed but not significant. A trend was observed for the amount of FP DNA, which was higher in reduced tillage fields compared with ploughed fields (data not shown).

Additional chopping of crop residues was only performed if the previous crop was cereal or maize. In these samples, the additional chopping did not have a significant effect on toxin contents or fungal DNA in any of the three years (data not shown). In 2013, we observed a tendency of higher values of FP and FL DNA as well as NIV and T-2/HT-2 in samples from fields with previous crop cereal without additional chopping compared with previous crop cereal and additional chopping. The same trend was observed for the combination with previous crop maize. In 2014 and 2015 however, the opposite trend was observed revealing higher values with additional chopping (data not shown).

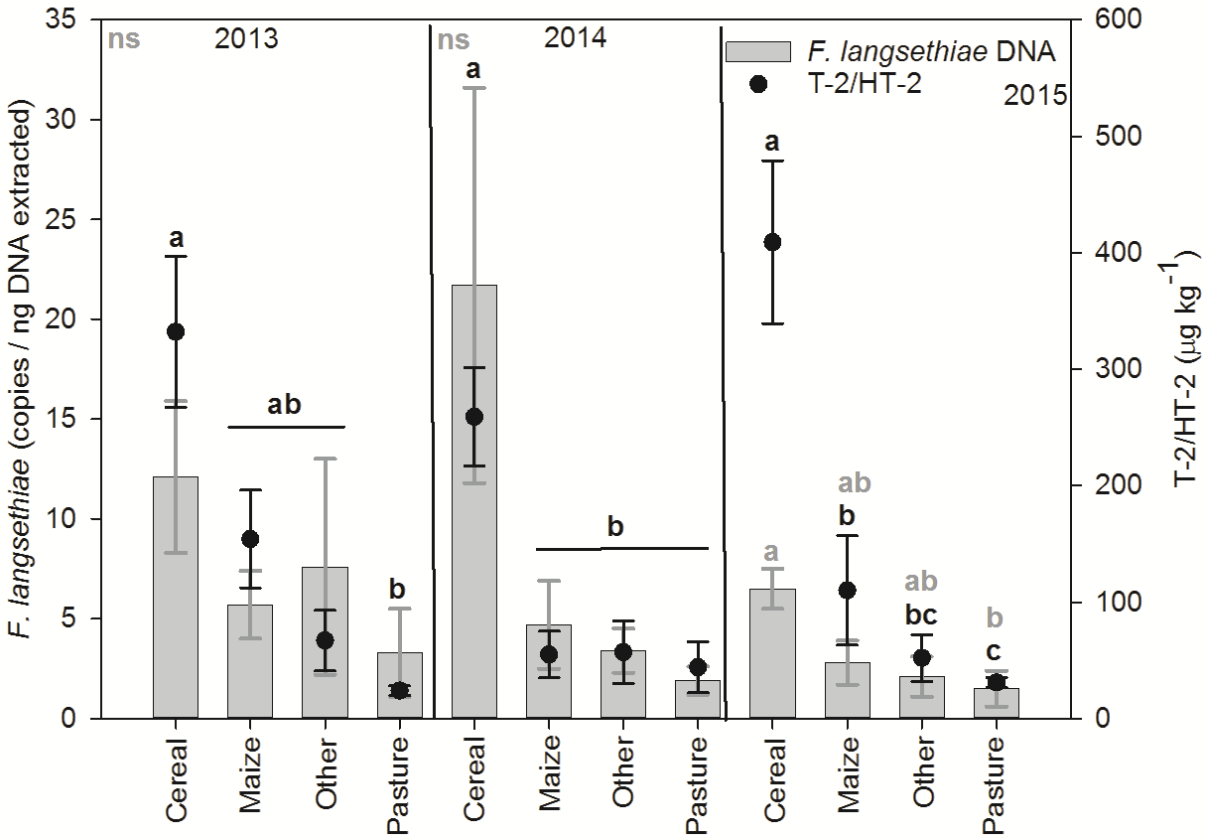

Figure 11: Association between the previous crop on T-2/HT-2 (µg kg^{-1}) content and *Fusarium langsethiae* (FL) DNA (copies/ng DNA extracted) in Swiss oats samples, collected in 2013 (n = 92), 2014 (n = 66) and 2015 (n = 167). Error bars represent the standard error of mean, means with the same letters are not significantly different for the respective year according to a Games-Howell post-hoc test at $\alpha = 0.05$. ns = not significant. Grey and black letters represent significant differences for the FL DNA amount and the T-2/HT-2 content, respectively. Meanings in cropping factors are as explained in the main text.

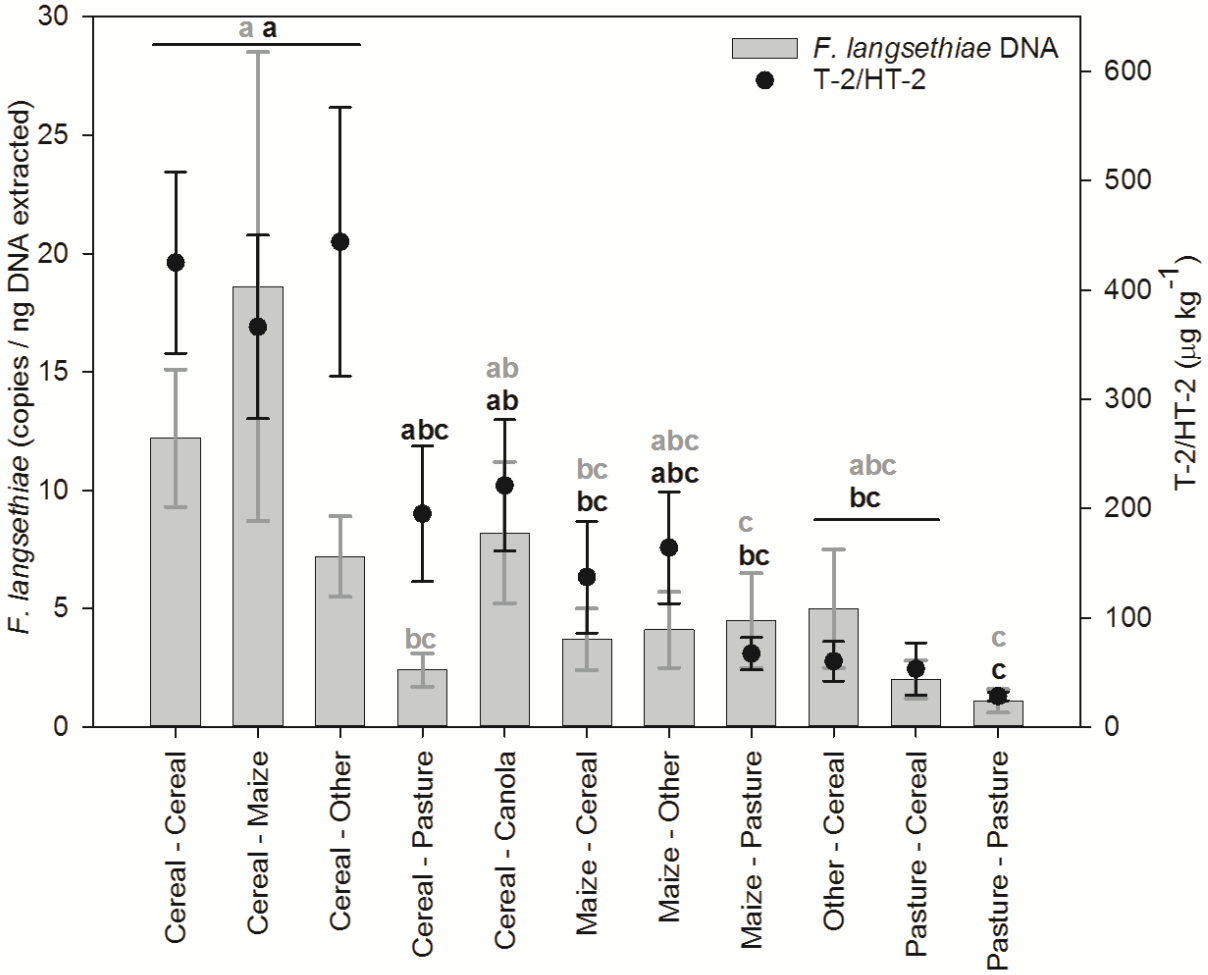

Figure 12: Association between the previous two crops on T-2/HT-2 (µg kg^{-1}) content and *Fusarium langsethiae* (FL) DNA (copies/ng DNA extracted) in Swiss oats samples, collected in 2013 to 2015, n = 308. Error bars represent the standard error of mean, means with the same letters are not significantly different according to a Tukey-Kramer test for interactions at $\alpha = 0.05$. Grey and black letters represent significant differences for the FL DNA amount and the T-2/HT-2 content, respectively. Meanings in cropping factors are as explained in the main text.

Table 12: Association between tillage and the interaction of previous crop x tillage on *Fusarium langsethiae* (FL) DNA and T-2/HT-2 content in Swiss oat samples collected between 2013 and 2015. n = number of samples; SEM = standard error of mean. Means with the same letters are not significantly different according to a Games-Howell test and a Tukey-Kramer test for interactions both at $\alpha = 0.05$. ns = not significant. Meanings of cropping factors are explained in the main text.

Cropping factor	n	FL (copies/ng DNA extracted)	T-2/HT-2 (µg kg^{-1})
Tillage		Mean (SEM)	
Reduced tillage	63	13.0 (6.3) ns	323 (69) a
Plough	262	7.0 (1.0) ns	240 (31) b
Previous crop + tillage		Mean (SEM)	
Cereal + reduced	38	18.3 (10.3) a	410 (103) a
Cereal + ploughed	163	9.2 (1.5) ab	348 (47) a
Maize + reduced	16	6.3 (2.4) abc	265 (106) ab
Maize + ploughed	54	3.8 (1.0) c	78 (13) bc
Other + reduced	4	3.6 (2.7) abc	66 (23) abc
Other + ploughed	19	4.0 (1.9) abc	56 (17) bc
Pasture + reduced	5	1.6 (1.0) bc	57 (36) abc
Pasture + ploughed	26	1.9 (0.8) c	29 (4) c

Association between the variety and nivalenol and T-2/HT-2

The varieties were grouped according to their occurrence as follows: Canyon (n=32), Gaillette (n=20), Husky (n=18), President (n=11), Triton (n=92), Wiland (n=105) or 'Other' (n=47) (eleven different varieties with fewer than ten samples each). Differences between the varieties were observed in 2013 and 2015, but not in 2014. Because of multicollinearity, this factor was removed from the model and no statistical analysis was performed. Nevertheless, we observed higher means of NIV and T-2/HT-2 contents in winter sown oat samples (168 µg kg^{-1}, 525 µg kg^{-1}, respectively) compared with spring sown oats (100 µg kg^{-1}, 122 µg kg^{-1}) in all three years, except for NIV in 2014.

Relation between the growing period, fungal DNA and mycotoxins

The calculation of the growing period was determined by the sowing and harvesting dates indicated by farmers. We observed no significant effects of the growing period on fungal DNA and toxin contents. A slightly higher T-2/HT-2 content in the samples with longer growing periods was observed.

Association between growth regulators, fungal DNA and mycotoxins

Growth regulators were used by 19% of the farmers and because the trend was similar in all three years, samples were pooled for statistical analysis. We observed a significant ($p=0.02$) lower mean FP DNA content (11 copies ng^{-1} DNA extracted) in samples where growth regulators were used compared with no use of growth regulators (17 copies ng^{-1} DNA extracted). The same trend was observed for NIV and T-2/HT-2 concentrations. The trend for the FL DNA content was contrary, revealing a higher content with the use of growth regulators.

Harvest quality

The harvest quality in kg per hectolitre was not significantly different between the respective years. It was slightly higher in 2015 (mean of 45.4 kg hl^{-1}) compared with 2013 and 2014 (both 42.1 kg hl^{-1}). No correlation between mycotoxin content (NIV, T-2/HT-2) or fungal DNA and harvest quality was observed.

Relation between weather conditions and mycotoxin contamination

Because not all farmers indicated the flowering period, we estimated those periods based on the sowing date and geographic origin of samples. Six weather stations (Bern, La Chaux-de-Fonds, Nyon, Payerne, Schaffhausen and Wynau) were used, because we received in all three years samples from these regions. Overall, the relation between weather conditions and FP or FL incidence and NIV or T-2/HT-2 contamination remains unclear. In 2015, the year with the highest average T-2/HT-2 contents (295 µg kg^{-1}), generally had more precipitation (18-101 mm) during May and June, which is the approximated stage of booting and flowering, compared with the long-time averages from 1981-2010 (see Table 18 in the supplementary material (chapter 3.8)). The approximated growth stage of dough development and ripening is July and August, were mostly less precipitation (16-67 mm) was observed in 2015 compared with the normal values from 1981-2010 (see Table 18 in the supplementary material (chapter 3.8)) (MeteoSwiss 2017). However, no clear influence of weather conditions during calculated flowering periods on fungal incidence and toxins was detected.

3.5 Discussion

In the current study, we investigated the *Fusarium* species and mycotoxin complex occurring in oat grains from Swiss farmers over three consecutive years. *Fusarium poae* (FP) and *F. langsethiae (*FL) are frequently isolated from oats (Edwards et al. 2012; Fredlund et al. 2013; Hofgaard et al. 2016a). In our study, FP was by far the dominant species isolated from oats, whereas the second most frequently occurring species differed in the individual years. In barley, the main species occurring in Switzerland is *F. graminearum* (FG) followed by *F. avenaceum* (FA) and FP (refer to chapter 2.4). This suggests that the different cereal types bear a different *Fusarium* species spectrum. Thus, depending on the main species, distinct control measures and resistance breeding for the specific cereal types and their *Fusarium* species may need to be established.

The mycotoxin with the highest occurrence and concentrations was T-2/HT-2 followed by NIV which is consistent with other reports showing high values of these toxins in oats (Hofgaard et al. 2016a; Edwards et al. 2009; Fredlund et al. 2013). In Europe, the predominant T-2/HT-2 producer is FL while *F. sporotrichioides* occurs only occasionally (Edwards et al. 2012; Bernhoft et al. 2010; Kosiak et al. 2003) and was also nearly absent in the current study. The strong correlation between T-2/HT-2 contamination and FL DNA amount in our samples indicates that FL is the prevalent producer in Switzerland. This was also reported in Sweden and Norway, as a strong correlation of FL and T-2/HT-2 was observed in these countries (Hofgaard et al. 2016a; Fredlund et al. 2013). Therefore, the occurrence of FL in oats and other small grain cereals should be monitored to elucidate whether this species will increase over the next years. Because of the high occurrence of FP, we expected a higher contamination with NIV than with T-2/HT-2. The low incidence of FL on agar plates but higher occurrence with qPCR indicates that qPCR is a more confidential test for this *Fusarium* species. We observed at least two-thirds less FL DNA than FP DNA, hence we assume that either FL is a very strong toxin producer or it was competing with other fungal species for space and nutrients. Co-inoculation experiments with FL and other *Fusarium* and *Microdochium* species could help to understand whether the toxin production was enhanced as a result of competition, as done by Siou et al. (2015) and Xu et al. (2007b). Also, climatic factors such as humidity or temperature could have stimulated toxin production and are not yet fully understood. In a climate chamber experiment with durum wheat, the optimum temperature for T-2/HT-2 production of FL was shown to be at 15 °C, which equals normal conditions during European summers (Nazari et al. 2014). A higher FL DNA amount and T-2/HT-2 content was reported after inoculating oat

plants at 10 °C under controlled conditions, compared with inoculation conditions at 15 °C and 20 °C (refer to chapter 5.4). This indicates, that FL is favoured by lower temperature conditions. We observed levels of NEO above the LOD only in samples with a T-2/HT-2 contamination with more than 200 µg kg^{-1}. Hence, we assume that NEO was also produced by FL as reported from strains isolated from Italian wheat (Lattanzio et al. 2013). However, the observed correlation in our study must be interpreted with caution, because NEO was only detected in a low number of samples. To determine whether there are different FL chemotypes, such as mainly T-2/HT-2 or NEO producers, and which conditions might promote their occurrence, the construction of a database would be useful, as recently established for the 15-AcDON, 3-AcDON and NIV chemotypes of FG and *F. culmorum* (Pasquali et al. 2016). Additionally, knowledge about factors which enhance the persistence or establishment of a specific chemotype can be achieved.

As observed in other studies (Edwards 2009; Hofgaard et al. 2016a), no correlation between the occurrence of T-2/HT-2 and DON was detected. This implies that the epidemiology of FL and FG might be different and that these two species have distinct environmental requirements. This phenomenon should be further investigated by monitoring the respective species and mycotoxins to see whether an annual variability reflecting different weather conditions exists.

Because of the characteristic *Fusarium* species spectrum of oats, the mycotoxin spectrum is also different compared with that of barley, where DON was the predominant mycotoxin and NIV and T-2/HT-2 were nearly absent (refer to chapter 2.4). This shows that analysis of cereals for several mycotoxins rather than a single one should be conducted to evaluate potential health risks.

The understanding of agronomic factors that affect the occurrence of the different *Fusarium* species and mycotoxins was a key aspect of this study. The major factor influencing T-2/HT-2 contamination was growing cereals as the previous crop. A higher contamination with T-2/HT-2 from fields with cereals as the previous crop was also observed by Edwards et al. (2009) and Bernhoft et al. (2012) in oats and by Orlando et al. (2010) in barley. We suggest that cereal straw is a preferred amplification substrate for FL and might play an important role in its life cycle. Nevertheless, low levels of FL DNA were found in cereal residues in a straw tillage experiment in Norway (Hofgaard et al. 2016b). Experiments on the survival of FL on different crop residues could help to identify the preferred host plant. Furthermore, the nutrient composition of crop residues, such as the carbon - nitrogen ratio, should be investigated as an influencing factor.

The lower occurrence of T-2/HT-2 in samples from ploughed fields compared with reduced tillage fields, independent of the previous crop, was also observed in other European countries (Edwards et al. 2009; Bernhoft et al. 2012; Parikka et al. 2007). In our study, the same outcome was observed for the NIV content in 2013. As ploughing reduces the amount of crop residues, we hypothesise that this leads to limited availability of colonisation space and nutrition source for *Fusarium* species. In addition, buried fungal inoculum might be degraded by other soil microorganisms (Wegulo 2012). Because the inoculum source of FL and FP is unknown, further studies should aim to determine their origin and reservoir as well as their overwintering strategies.

In winter sown oat samples, concentrations of NIV and T-2/HT-2 were higher compared with those of spring sown oat samples, which matches results from a monitoring study in the UK (Edwards et al. 2009) and was also observed during a field experiment (Suproniene et al. 2010). The reason for this finding remains unclear but could reflect a longer vegetation period. However, in our study, a longer vegetation period led only to slightly higher contents of T-2/HT-2 and showed no influence on the NIV content. Although the latter might be a consequence of the overall low NIV concentrations, we assume that a longer potential colonisation time for the fungus probably does not result in a higher toxin accumulation. Delayed harvest could be a further factor that enhances mycotoxin content, but it did not show an effect on DON in wheat (Xue et al. 2004). The differences between winter and spring varieties might also be explained by different levels of resistance as opposed to the sowing time. Experiments with different varieties, sowing and harvesting times might contribute to develop oat cropping systems with low FL infections and less T-2/HT-2 accumulation.

The FP incidence and contamination with NIV and T-2/HT-2 was higher in samples where no growth regulators were applied. In agreement with our results, Mankevičiene et al. (2008) observed a higher diversity of potential T-2 producers in winter wheat that was not treated with growth regulators. This finding was unexpected because other studies reported a higher incidence of FG and DON in wheat when growth regulators were applied (Maji and Imolehin 2002). They assumed that the shortened plants were closer to the ground, which favours infection from ejected ascospores. Furthermore, Bernhoft et al. (2012) assumed that plants treated with growth regulators become bushier, which enhances the humidity and spread of *Fusarium* spores. The absence of growth regulators increases the risk of lodging and might lead to higher contamination with NIV and DON (Nakajima et al. 2008). Still, this factor was not considered in our study but should be investigated in the field with respect to T-2/HT-2 contents. The contrasting results might be due to different host and environmental requirements

of FG and FL. Because of the cryptic life cycle of FL, it is unknown whether it produces ascospores. In addition, the morphology of the oat panicle, where branches arise from the main axis bearing spikelets consisting of two or three florets at their tips, is different to the wheat and barley ear, which consists of a central stem with rows of flowers grouped closely together (Welch 1995; Schlömer et al. 1960). These differences might result in dissimilar microclimates for fungal spores. Consequently, the mode of FL infection in oats and wheat should be investigated to understand the role of the reproductive organ.

Higher concentrations of NIV or T-2/HT-2 or higher incidences of FP and FL did not have a significant effect on the harvest quality. Nevertheless, an economic loss caused by rejection or downgrading because of high mycotoxin levels may occur, in particular if the current indicative level for T-2/HT-2 concentrations (The European Commission 2013) will be turned into legally binding maximum limits. Because FL and FP infections usually do not lead to visual symptoms on the kernels (Torp and Adler 2004; Stenglein 2009), healthy looking grains may still contain elevated amounts of mycotoxins and therefore, oat producers and collection centres have to rely on mycotoxin analyses.

The principal parameters for a *Fusarium* infection are weather conditions during anthesis. We observed the highest T-2/HT-2 contents in 2015, with comparably more precipitation during the estimated flowering period but less precipitation during the estimated period of fruit development. Thus, we hypothesise that the drier conditions during grain development in 2015 favoured T-2/HT-2 production by FL. We assume that the dry conditions were less favourable for spread, infection during anthesis and colonisation by other *Fusarium* species, leading to more space and nutrients for the development of FL. This hypothesis can be supported by the results of Xu et al. (2013), who observed a positive correlation of T-2/HT-2 with warm and wet conditions before estimated anthesis and dry conditions in the following period.

The NIV contents were also higher in 2015, which suggests that preferred weather conditions of FL might meet those of FP. In wheat, an enhanced incidence of FP was shown with increasing wetness duration and temperature during artificial inoculation at mid-anthesis (Xu et al. 2007a). Currently, we conduct epidemiological studies with FL and FP in oats to better understand the climatic factors which promote the infection and mycotoxin accumulation in grains.

3.6 Conclusions and Outlook

This three year *Fusarium* monitoring on Swiss commercially grown oats clearly showed a high incidence of FP and of the mycotoxins T-2/HT-2, which are mainly produced by FL. The obtained results underline the fact that an observed fungal incidence by itself is not sufficient to estimate subsequent mycotoxin accumulation. This study is unique because it described both the current occurrence of FHB causing fungi and their mycotoxins, and also identified the effect of cropping techniques on fungal incidence and toxin contamination in oat grains. The analysis of the prevailing cropping factors revealed 1) higher T-2/HT-2 contaminations in oat samples from fields where cereals were the previous crop; 2) higher amounts of NIV and T-2/HT-2 in samples from fields with reduced tillage compared with those from ploughed fields; and 3) higher levels of T-2/HT-2, NIV and FP in autumn sown oat varieties compared with spring sown oat varieties. Based on these findings, crop rotations with cereals before oats, especially in reduced tillage systems, should be avoided. Because the outcomes of this study are based on oat fields with vastly different backgrounds in terms of cropping system and prevailing weather, long-term field experiments are needed to evaluate the susceptibility of the currently available oat varieties. Further monitoring on the occurrence and shift of the entire *Fusarium* spectrum should be continued to observe potential correlations with prevailing weather conditions. Since the co-contamination patterns in oat samples were highly diverse, ranging from high levels of T-2/HT-2 and low levels of DON and NIV, vice versa to high levels of DON and low levels of NIV, we assume that the respective fungal species have different environmental requirements. The newly established tolerable intake and acute reference dose values for T-2/HT-2 established by the EFSA (EFSA 2017) shows a rising concern about these mycotoxins. Despite the current lack of maximum levels for T-2/HT-2 and NIV, oats for human consumption should be tested for these highly toxic contaminants to ensure food safety and to evaluate whether there is a need for establishing maximum limits by the European Commission. Currently, epidemiological studies are conducted to investigate the overwinter survival of FP and FL, which will not only help to uncover the potential life cycle of these neglected toxigenic species but also to develop recommendations for a sustainable and efficient FHB control in oats.

3.7 Acknowledgements

The authors thank the Swiss cantonal plant protection officers who provided addresses of oat producers and all participating farmers for kindly providing samples of their harvest and for completing the questionnaires. Excellent technical support was provided by Irene Bänziger, Fionna Loeu, Andreas Kägi and Georg Oberer.

This work was funded by the Swiss National Science Foundation in the frame of the national research programme 'Healthy Nutrition and Sustainable Food Production' (NRP 69) [grant number 406940_145210].

No potential conflict of interest was reported by the authors.

3.8 Supplementary Material

Table 13: Number of samples received from the respective canton in 2013-2015.

Canton	2013	2014	2015
Bern	6	7	22
Basel	5	6	14
Fribourg	18	9	50
Geneva	3	1	3
Grisons	0	0	1
Jura	2	2	2
Lucerne	8	5	7
Neuchâtel	6	2	12
St Gallen	0	1	0
Schaffhausen	13	16	7
Solothurn	6	11	0
Thurgau	16	1	3
Vaud	9	4	38
Zug	0	0	1
Zurich	0	1	7

Table 14: Summary of multiple linear regression for variables predicting *Fusarium poae* (FP) DNA, *F. langsethiae* (FL) DNA, Nivalenol (NIV) content and T-2/HT-2 content of the years 2013-2015; n = 325; R^2 = predictive performance of the model.

		FP DNA		FL DNA		NIV		T-2/HT-2	
Variable	Degrees of freedom	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
tillage	1	0.013	0.910	2.975	0.086	2.870	0.091	5.309	0.022
Previous crop	3	3.637	0.013	11.743	<0.001	0.671	0.414	84.084	<0.001
Pre previous crop	4	0.830	0.507	1.851	0.119	0.093	0.760	3.131	0.078
Additional chopping	1	0.299	0.585	0.039	0.843	1.396	0.238	0.583	0.446
Growth regulators	1	5.330	0.022	0.033	0.856	3.285	0.071	0.683	0.409
Previous crop + tillage	2	0.898	0.443	0.203	0.894	0.285	0.594	0.022	0.883
Previous crop + Pre previous crop	9	1.389	0.246	1.662	0.175	0.439	0.508	1.642	0.201
Previous crop + additional chopping	2	0.194	0.824	0.895	0.410	1.697	0.194	6.176	0.014
R^2		0.09		0.17		0.03		0.24	

Table 15: Summary of multiple linear regression for variables predicting *Fusarium poae* (FP) DNA and *F. langsethiae* (FL) DNA, Nivalenol (NIV) content and T-2/HT-2 content of the year 2013; n = 92; R^2 = predictive performance of the model.

		FP DNA		FL DNA		NIV		T-2/HT-2	
Variable	Degrees of freedom	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
tillage	1	0.020	0.889	1.228	0.271	13.868	<0.001	2.297	0.133
Previous crop	3	0.452	0.503	7.533	0.007	0.050	0.824	16.004	<0.001
Pre previous crop	4	0.004	0.948	6.517	0.013	0.233	0.631	2.203	0.142
Additional chopping	1	0.301	0.585	0.142	0.708	3.346	0.071	0.037	0.847
Growth regulators	1	5.246	0.025	0.098	0.755	1.131	0.291	0.098	0.756
Previous crop + tillage	2	0.092	0.763	0.082	0.775	0.486	0.487	0.223	0.638
Previous crop + Pre previous crop	9	0.975	0.326	0.007	0.935	0.415	0.521	0.324	0.571
Previous crop + additional chopping	2	0.096	0.757	3.022	0.086	0.458	0.501	4.912	0.029
R^2		0.08		0.18		0.19		0.24	

Table 16: Summary of multiple linear regression for variables predicting *Fusarium poae* (FP) DNA and *F. langsethiae* (FL) DNA, Nivalenol (NIV) content and T-2/HT-2 content of the year 2014; n = 66; R^2 = predictive performance of the model.

		FP DNA		FL DNA		NIV		T-2/HT-2	
Variable	Degrees of freedom	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
tillage	1	0.346	0.559	1.460	0.232	0.452	0.504	1.721	0.195
Previous crop	3	1.862	0.178	8.377	0.005	0.688	0.411	24.858	<0.001
Pre previous crop	4	0.753	0.389	3.105	0.083	0.846	0.362	0.121	0.729
Additional chopping	1	0.851	0.360	0.142	0.708	0.211	0.648	1.817	0.183
Growth regulators	1	0.150	0.700	1.267	0.265	3.495	0.067	0.686	0.411
Previous crop + tillage	2	0.394	0.533	0.575	0.452	0.159	0.692	0.328	0.569
Previous crop + Pre previous crop	9	3.691	0.060	0.015	0.903	1.843	0.180	0.850	0.360
Previous crop + additional chopping	2	0.519	0.474	0.831	0.366	0.003	0.958	3.262	0.076
R^2		0.13		0.22		0.12		0.37	

Table 17: Summary of multiple linear regression for variables predicting *Fusarium poae* (FP) DNA and *F. langsethiae* (FL) DNA, Nivalenol (NIV) content and T-2/HT-2 content of the year 2015; n = 167; R^2 = predictive performance of the model.

		FP DNA		FL DNA		NIV		T-2/HT-2	
Variable	Degrees of freedom	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
tillage	1	0.090	0.765	0.484	0.488	1.357	0.246	1.178	0.279
Previous crop	3	3.002	0.085	4.870	0.029	0.316	0.575	42.798	<0.001
Pre previous crop	4	0.433	0.512	0.809	0.370	0.568	0.452	0.570	0.452
Additional chopping	1	0.784	0.377	0.296	0.587	1.684	0.196	0.426	0.515
Growth regulators	1	2.034	0.156	0.314	0.576	0.853	0.357	0.145	0.704
Previous crop + tillage	2	2.459	0.119	1.451	0.230	0.111	0.739	0.005	0.946
Previous crop + Pre previous crop	9	0.201	0.655	1.673	0.198	0.091	0.763	0.728	0.395
Previous crop + additional chopping	2	0.475	0.492	0.393	0.532	2.954	0.088	0.680	0.411
R^2		0.06		0.06		0.05		0.23	

Table 18: Sum of precipitation [mm] in the months April-August of the years 2013-2015, standard value of the years 1981-2010 and deviation.

Year	Month	Location	Precipitation [mm]	Precipitation Standard value 1981-2010 [mm]	Deviation [mm]
2013	April	Bern	116	82	+ 34
	May	Bern	117	119	- 2
	June	Bern	103	111	- 8
	July	Bern	107	106	+ 1
	August	Bern	57	116	- 59
2013	April	Chaux de Fonds	121	99	+ 22
	May	Chaux de Fonds	195	141	+ 54
	June	Chaux de Fonds	146	124	+ 22
	July	Chaux de Fonds	119	126	- 7
	August	Chaux de Fonds	69	136	- 67
2013	April	Nyon	94	67	+ 27
	May	Nyon	121	86	+ 35
	June	Nyon	80	83	- 3
	July	Nyon	149	78	+ 71
	August	Nyon	47	78	- 31
2013	April	Payerne	92	68	+ 24
	May	Payerne	115	90	+ 25
	June	Payerne	57	90	- 33
	July	Payerne	90	88	+ 2
	August	Payerne	69	95	- 26
2013	April	Schaffhausen	111	64	+ 47
	May	Schaffhausen	148	88	+ 60
	June	Schaffhausen	54	92	- 38
	July	Schaffhausen	106	95	+ 11
	August	Schaffhausen	29	86	- 57
2013	April	Wynau	108	76	+ 32
	May	Wynau	127	107	+ 20
	June	Wynau	70	106	- 36
	July	Wynau	119	110	+ 9
	August	Wynau	49	119	- 70
2014	April	Bern	87	82	+ 5
	May	Bern	126	119	+ 7
	June	Bern	67	111	- 45
	July	Bern	252	106	+ 146
	August	Bern	81	116	- 35
2014	April	Chaux de Fonds	95	99	- 4
	May	Chaux de Fonds	177	141	+ 36
	June	Chaux de Fonds	72	124	- 52
	July	Chaux de Fonds	339	126	+ 213
	August	Chaux de Fonds	148	136	+ 12
2014	April	Nyon	48	67	- 19
	May	Nyon	74	86	- 12
	June	Nyon	72	83	- 11

	July	Nyon	174	78	+ 96
	August	Nyon	84	78	+ 6
2014	April	Payerne	62	68	- 6
	May	Payerne	84	90	- 7
	June	Payerne	42	90	- 48
	July	Payerne	193	88	+ 105
	August	Payerne	89	95	- 6
2014	April	Schaffhausen	81	64	+ 17
	May	Schaffhausen	31	88	- 57
	June	Schaffhausen	47	92	- 45
	July	Schaffhausen	150	95	+ 55
	August	Schaffhausen	79	86	- 8
2014	April	Wynau	89	76	+ 13
	May	Wynau	86	107	- 22
	June	Wynau	83	106	- 23
	July	Wynau	222	110	+ 112
	August	Wynau	90	119	- 29
2015	April	Bern	69	82	- 13
	May	Bern	149	119	+ 30
	June	Bern	75	111	- 36
	July	Bern	39	106	- 67
	August	Bern	79	116	- 37
2015	April	Chaux de Fonds	122	99	+ 23
	May	Chaux de Fonds	159	141	+ 18
	June	Chaux de Fonds	150	124	+ 26
	July	Chaux de Fonds	83	126	- 44
	August	Chaux de Fonds	68	136	- 68
2015	April	Nyon	55	67	- 12
	May	Nyon	121	86	+ 35
	June	Nyon	55	83	- 29
	July	Nyon	62	78	- 16
	August	Nyon	99	78	+ 21
2015	April	Payerne	56	68	- 12
	May	Payerne	109	90	+ 19
	June	Payerne	72	90	- 18
	July	Payerne	25	88	- 63
	August	Payerne	79	95	- 16
2015	April	Schaffhausen	61	64	- 3
	May	Schaffhausen	150	88	+ 62
	June	Schaffhausen	134	92	+ 42
	July	Schaffhausen	33	95	- 62
	August	Schaffhausen	64	86	- 22
2015	April	Wynau	76	76	± 0
	May	Wynau	208	107	+ 101
	June	Wynau	128	106	+ 22
	July	Wynau	47	110	- 63
	August	Wynau	62	119	- 57

The previous two chapters focused on the main occurring *Fusarium* species and mycotoxins in barley and oats as well as influencing agronomic measures. FHB is a re-occurring disease, hence, to predict potential contaminations of harvested grains, it is essential to elucidate weather conditions, such as temperature and precipitation, which affect infection of the main *Fusarium* species. These data can subsequently be used to develop forecasting systems as decision tools with respect to fungicide applications. In the following two chapters, emphasis is laid on epidemiological studies conducted in the climate chamber and in the field to examine weather conditions that are favourable for an infection with *F. graminearum* in barley and for *F. poae* and *F. langsethiae* in oats.

4 The required infection conditions of *Fusarium graminearum* in barley are different than those in wheat

Submitted to European Journal of Plant Pathology

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4.1 Abstract

Fusarium head blight is one of the most noxious cereal diseases and is caused by different *Fusarium* species. Worldwide, *F. graminearum* (FG) is the most dominant in barley and wheat. Barley is mostly used as on farm fodder and thus routine mycotoxin analyses similar to those of cereals for human consumption is not performed. Hence, an early prediction of mycotoxin levels is important for farmers to minimise the risk of contaminated feed but also of contaminated cereals entering the cereal supply chain. Climate chamber experiments with artificial FG infection of barley investigating the influence of different temperatures (10 °C, 15 °C, 20 °C) and durations (4 h, 8 h, 12 h) at 99% relative humidity (rH) revealed a higher contamination with deoxynivalenol (DON) in the 15 °C treatments and higher contaminations after prolonged humidity duration for the 20 °C treatments. The higher contamination of the barley grains at 15°C is different from the warmer infection requirements known for FG in wheat. In addition, field experiments where spore deposition during anthesis as well as disease incidence, fungal amount and mycotoxins were observed, showed that the overwintered straw resulted, depending on the year, in a higher FG incidence and DON content in the harvested

barley grains. The obtained knowledge, together with influencing cropping factors, will be used to extend the existing forecasting model FusaProg for DON in wheat towards barley to predict the potential mycotoxin contamination in harvested grains.

4.2 Introduction

Fungi of the genus *Fusarium* can cause Fusarium head blight (FHB), one of the most noxious cereal diseases. In Switzerland, *F. graminearum* (FG) is the most occurring species in barley and wheat (refer to chapter 2.4; Vogelgsang et al. 2011) and the typical symptoms are discoloured and shrivelled grains (Parry et al. 1995). Apart from reduced grain quality and yield, FG produces mycotoxins such as deoxynivalenol (DON) (Bottalico and Perrone 2002; Osborne and Stein 2007). Mycotoxins are harmful to humans and animals and thus the European Commission has set a maximum level for unprocessed barley used for human consumption of 1'250 µg kg^{-1} (The European Commission 2006), which was adopted in the respective Swiss legislation.

In Switzerland, barley is mainly grown as fodder but also used for human consumption as malt and beer or for baking and cooking purposes. Fodder barley is generally used directly on farm and thus not regularly analysed compared with cereals used for human consumption which can result in undetected, elevated mycotoxin concentrations. Pigs fed with DON contaminated barley showed a reduced feed intake, diarrhoea and vomiting (reviewed in D'Mello et al. 1999). Since FHB epidemics are strongly dependent on prevailing weather conditions, a control with fungicides is not always required (McMullen et al. 1997; Brustolin et al. 2013). Hence, an application according to a forecasting system can avoid unnecessary fungicide applications (Wegulo et al. 2015).

Knowledge about the most influencing cultivation measures is important to reduce the risk of a FG infection in barley and was assessed during the last years (refer to chapter 2). In addition, data about the most susceptible growth stages and favourable infection conditions, which were shown to affect the infection with FG, are necessary to predict the occurrence in a particular field (Cowger et al. 2009; Brustolin et al. 2013). Temperature and precipitation are also embedded in several forecasting systems such as FusaProg in Switzerland (Musa et al. 2007), DONCast in Canada (Schaafsma and Hooker 2007) and Qualimètre® in France and Belgium (Froment et al. 2011). However, they all focus on wheat or maize and until to date, no forecasting system for FHB in barley is available. Models on FHB incidence in barley were

tested in China since the 1980s, although only for a particular cultivar and region (cited in Choo 2009).

Hence, the main objectives of these studies were (I) to assess the most critical temperature and moisture conditions for an FG infection in barley under controlled conditions and (II) to observe the FG spore deposition during the anthesis period in relation to the prevailing weather conditions in the field.

4.3 Material and Methods

Inoculum preparation

Three FG strains, all isolated from Switzerland and known to produce DON, were chosen. The geographic origin and host from which the used strains were derived are indicated in Table 20 in the supplementary material (chapter 4.8). The inoculum preparation for the climate chamber experiment and the straw used in the field experiment was conducted as described in chapter 5.3).

Climate chamber experiment

Two spring barley varieties, Ascona and Concerto, were used to study the influence of temperature and the duration of 99% relative humidity (rH). The uncoated seeds were put into germination boxes lined with three wet filter papers (150 x 130 mm; Type 614, Macherey-Nagel, Düren, Germany) and incubated for 36 h at 5 °C in the dark. Subsequently, the boxes were incubated at 18 °C for 72 h in an incubator (RUMED, Rubarth Apparate GmbH, Laatzen, Germany) to ensure an equal development of the plants. Three germinated seedlings were planted in pots (ø 15 cm, 13 cm height) in standard soil (Oekohum GmbH, Herrenhof, Switzerland) at a depth of two to three cm. Climate chamber conditions were 18 °C / 75% rH during the day and 13 °C / 85% rH during the night for the first 14 days, then 20 °C / 70% rH (day) and 15 °C / 80% rH (night) until decimal code (DC) 83 (Zadoks et al. 1974). The conditions were then set to 22 °C / 70% rH (day) and 18 °C / 70% rH (night) until harvest. Each pot was fertilised from DC 41 until DC 83 with 250 ml of a 1% dilution Wuxal® P Profi (Manna, Düsseldorf, Germany).

During anthesis (DC 65), three spikes per pot were spray inoculated using a pressure of 2 bar with 30 ml spore suspension adjusted to 2 x 10^5 spores/ml containing the three different FG strains to mimic a population. Subsequently, the plants were transferred into a climate chamber

in the dark and the tested temperature and humidity duration combinations were: 10 °C / 4 h, 10 °C / 8 h, 10 °C / 12 h, 15 °C / 4 h, 15 °C / 8 h, 15 °C / 12 h, 20 °C / 4 h, 20 °C / 8 h and 20 °C / 12 h. A suspension using only 0.0125% Tween® water at 20 °C / 12 h served as a control treatment. Immediately after the respective incubation treatments, the plants were transferred into a climate chamber with 20 °C / 70% rH (day) and 15 °C / 80% rH (night).

Set up of the field experiment

Location

The field experiments were conducted at the Agroscope research institute in Zurich, Switzerland (N 047° 24 430; E 008° 30 597) over two years, 2015 and 2016. The previous crop in both years was potato.

Straw inoculation, plot preparation and field management

The straw inoculation for all three treatments (control, freshly inoculated and overwintered) was done as described in chapter 5.3 using a FG spore suspension as described above. For the "overwintered" treatment, barley straw from field experiments artificially inoculated during flowering with FG in the previous year was taken after harvest and stored at 5 °C in the dark.

Seedbed preparation was done by ploughing (September 2014/2015) followed by a grubber treatment and subsequent harrowing (both October 2014/2015). Sowing of the winter barley varieties Meridian and Semper (seed density 0.3 t/ha, row distance 15.5 cm), was done in October 2014 and 2015, respectively. The fertilisation and plant protection applications in the respective years are indicated in Table 21 in the supplementary material (chapter 4.8).

The experiment consisted of a randomised complete block design with four replicate blocks. The treatments (plot size 3 m x 9 m) comprised three different straw inoculation methods: I: autoclaved straw (control), II: freshly inoculated straw and III: overwintered straw. Next to the treatment plots where the straw was applied, a side plot with either Meridian or Semper of the same dimension (but without straw) was sown. These plots were additionally surrounded by triticale plots of 3 m x 12 m to minimise the risk of cross contamination. The straw was applied to the respective plots at DC 25 in 2015 and 2016, respectively. In each plot, 2.5 kg straw was applied.

All plots were harvested with a plot combine harvester end of July 2015 and beginning of August 2016, respectively. Weather data (daily mean temperatures, rH and sum of precipitation) during the entire cropping season were obtained from a nearby weather station operated by the Federal Office of Meteorology and Climatology, MeteoSwiss.

Spore traps

Observation of the spore deposition during the anthesis period was done with spore traps as described in chapter 5.3. After the incubation process, the different *Fusarium* species were identified based on their morphology and according to Leslie and Summerell (2006).

Fusarium graminearum disease rating

The disease rating in the climate chamber experiment was done by counts of visibly infected spikelets per spike of the three inoculated ears seven and 14 days post inoculation (dpi) and expressed in percent severity of the total number of spikelets per spike.

For the field experiment, the disease rating was performed twice by randomly selecting ten times ten ears of each plot. The first rating was performed once the first symptoms appeared (13 days after flowering (daf) at DC 73 in 2015, 18 daf (DC 73) in 2016) and the second rating followed after one more week (20 daf (DC 77) in 2015 and 25 daf (DC 77) in 2016). The number of spikelets from 100 randomly selected ears were counted and averaged to calculate the severity in percent.

Quantification of *Fusarium graminearum* by quantitative PCR

Quantitative PCR (qPCR) was performed to determine the amount of FG DNA in milled grain samples from the climate chamber experiment. The extraction and measurement of the DNA concentration was done as described in chapter 3.3. Due to the high amounts of FG DNA, the extracted samples were diluted 1:200 to be within the range of the standard curve. All reactions were analysed in a CFX96™ Real-Time PCR Detection System–IVD (Bio-Rad, Hercules, USA) in a 96-well plate format (Hard-shell full-height 96-well semi-skirted PCR plates, BioRad, Hercules, USA). In every performed assay, all standards and the negative control (double distilled water) were run as triplicates.

The plasmid needed to establish the standard curve was prepared according to instructions of the Promega pGem®-T and pGem®–T Easy Vector Systems Kit (Promega, Madison, USA) with *E. coli* DH5α. Plasmid DNA from *E. coli* DH5α recombinants was extracted and purified with the QIAprep® Miniprep Kit (Qiagen Scientific, Venlo, The Netherlands).

The amplification mix consisted of 0.4 µl primer pairs (Microsynth AG, Balgach, Switzerland) Fg16N F (5’ ACAGATGACAAGATTCAGGCACA 3’) and

Fg16N R (5’ TTCTTTGACATCTGTTCAACCCA 3’), 10 µl IQ SYBR® Green Supermix (Bio-Rad, Cressier, Switzerland) and 4.6 µl ddH_2O. The qPCR volume per well was 20 µl and

contained 15 µl amplification mix and 5 µl diluted sample DNA. The initial melting curve consisted of 3 minutes (min) at 95 °C followed by 30 seconds (sec) at 92 °C. For the subsequent 40 cycles, the plate was kept at 64 °C for 45 sec, then 45 sec at 72 °C, followed by 5 min at 72 °C.

Fusarium species incidence

Incidences of *Fusarium* species in barley grains from the field experiment were determined with the seed health test method described in Vogelgsang et al. (2008a) and identified as described above.

Mycotoxin measurement in harvested grains

Deoxynivalenol was measured using a competitive Enzyme Linked Immunoabsorbent Assay (ELISA), Ridascreen® FAST DON (R-Biopharm AG, Darmstadt, Germany). For the field experiment, sample preparation and ELISA assay was conducted according to the manufacturers' instructions. Because only the three inoculated ears per pot were harvested a reduced amount of samples was available for the climate chamber experiment. Hence, the extraction procedure was modified as follows: A sample volume of 70 mg flour was weighted into a two ml Eppendorf tube® and 1.4 ml demineralised water was added. The samples were vortexed for 10 sec and subsequently shaken on a horizontally shaker (Lab-Shaker Typ LSR-V, Birsfelden, Switzerland) at 250 rpm for 15 min at room temperature. Afterwards, the samples were centrifuged at 13.000 rpm for 5 min at room temperature in a table top centrifuge (Biofuge 13, Heraeus, Osterode am Harz, Germany). Three aliquots of each 350 µl supernatant were then pipetted into 1.2 ml collection microtubes (Qiagen, Hombrechtikon, Switzerland).
Samples absorbance were measured using a spectrometer (Tecan Sunrise™) and the toxin concentrations were estimated via a standard curve of known DON concentrations by Ridasoft Win 1.84 software (R-Biopharm AG). The limit of detection (LOD) was 222 µg kg^{-1}, individual values below the LOD were replaced by a constant value of 111 µg kg^{-1}.

Statistical analysis

For statistical analysis, the software R Version 3.3.2, R Studio Version 1.0.136 and the packages agricolae, nlme and lsmeans were used (R Core Team 2015; R Studio Team 2015; de Mendiburu 2015; Pinheiro et al. 2016; Lenth 2016). Data from both experiments were verified graphically for homogeneity of variances and normal distribution of residuals. All disease rating and seed health test data were arcsine square root transformed, whereas DON and DNA data were log transformed to meet the requirements for the post-hoc and correlation tests.

To investigate significant influencing factors, a linear mixed effects model was created followed by an analysis of variance (ANOVA). For the climate chamber experiment, the factors temperature, humidity duration and variety were set as fixed factors, whereas experiment repetition and block were set as random factors. For the field experiment, year, straw inoculation treatment and variety were set as fixed factors and block was set as random factor. Obtained significant factors were then examined using the Tukey method for pairwise comparison of least-square means ($\alpha = 0.05$).

Using the transformed data, Pearson correlation was calculated to determine the relationship between disease rating, qPCR and DON data for the climate chamber experiment and between disease rating, seed health test and DON data for the field experiment. For figures, the software SigmaPlot© Version 13.0.0.83 (Systat Software, Inc.) and Microsoft® Excel 2013 were used and untransformed data were plotted.

4.4 Results

Climate chamber experiment

The FG DNA amount and DON content was above the LOD in 94% and 98%, respectively, in the grains of all the inoculated plants. Neither DON nor FG DNA was measured in grains of the control treatment plants.

In general, the disease symptoms increased from the first to the second rating and only results from the first date are presented here. The temperature showed a highly significant effect on the disease severity ($p < 0.001$). Averaged over the humidity duration, harvested grains of variety Ascona and Concerto revealed the highest disease severity after an inoculation at 15 °C. For variety Ascona, the inoculation at 10 °C resulted in significantly lower disease severity compared with the 15 °C treatment ($p = 0.004$), whereas for variety Concerto, the 20 °C treatment led to significantly lower ($p = 0.021$) disease severity (Figure 13).

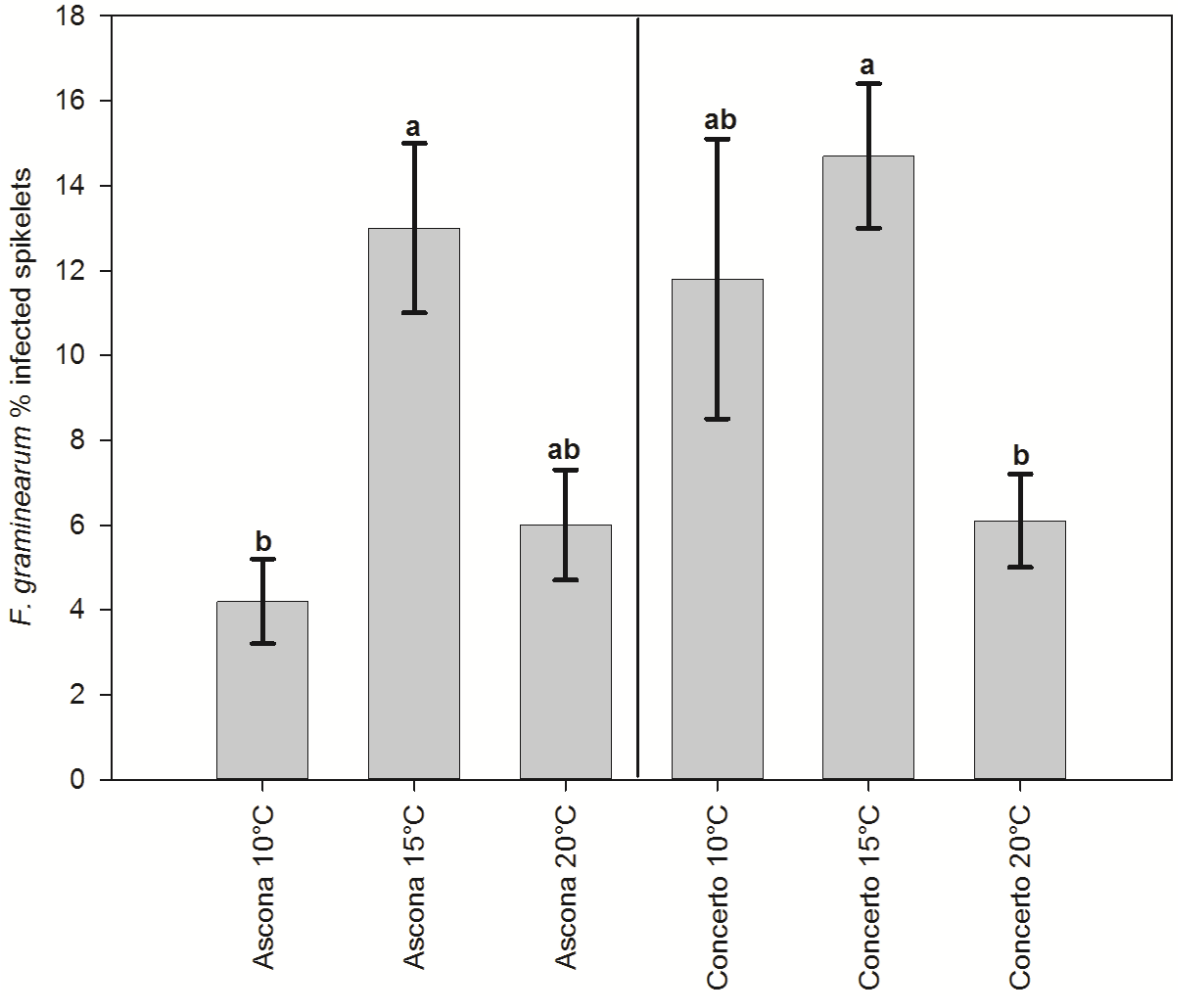

Figure 13: Effect of temperature and variety on *Fusarium graminearum* (FG) disease severity (%) in two barley varieties (Ascona, Concerto) based on the number of infected spikelets after 7 days post inoculation in a climate chamber. Pooled data from two experimental runs. n = 108. The statistical analysis was conducted for each variety separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$.

Averaged over the humidity duration, harvested grains from the variety Ascona inoculated at 15 °C contained significantly more FG DNA and DON compared with grains from plants inoculated at 10 °C ($p = 0.028$ and $p = 0.023$) and at 20 °C ($p = 0.035$ and $p = 0.017$). No differences in terms of the FG DNA amount and DON content between the temperatures was observed for the variety Concerto. Averaged over the temperatures, harvested grains from the variety Concerto inoculated for 12 h at 99% rH, showed a significantly higher DON content (22'290 µg kg^{-1}; $p = 0.012$) compared with grains from plants inoculated for 4 h (7'862 µg kg^{-1}). In both varieties, a higher FG DNA amount, DON contamination and severity was observed with increasing humidity duration for the 20 °C treatments (Figure 14; and Figure 19 in the supplementary material (chapter 4.8)). The highest FG DNA amount and DON content

for the variety Ascona were detected in grains from plants incubated at 15 °C and with 4 h 99% rH and for grains from the variety Concerto at 15 °C / 12 h 99% rH (Figure 14). With longer humidity durations, the DON contamination and severity was decreased in harvested grains from the variety Ascona at 10 °C (Figure 14; and Figure 19 in the supplementary material (chapter 4.8)), whereas the DON content was increased in harvested grains from the variety Concerto under all tested temperatures (Figure 14). In both varieties, no significant differences between the treatment combinations for any collected data was observed.

For the variety Ascona, a significant correlation between disease severity and FG DNA amount ($r = 0.41$; $p = 0.002$) as well as for disease severity and DON contamination ($r = 0.57$; $p < 0.001$) was observed. The correlation of FG DNA amount and DON content was also significant ($r = 0.47$; $p < 0.001$). The same was observed for the variety Concerto, which revealed correlations between the disease severity and FG DNA amount ($r = 0.36$; $p = 0.009$) and with the DON content ($r = 0.45$; $p < 0.001$) as well as between the FG DNA amount and the DON content ($r = 0.42$; $p = 0.002$).

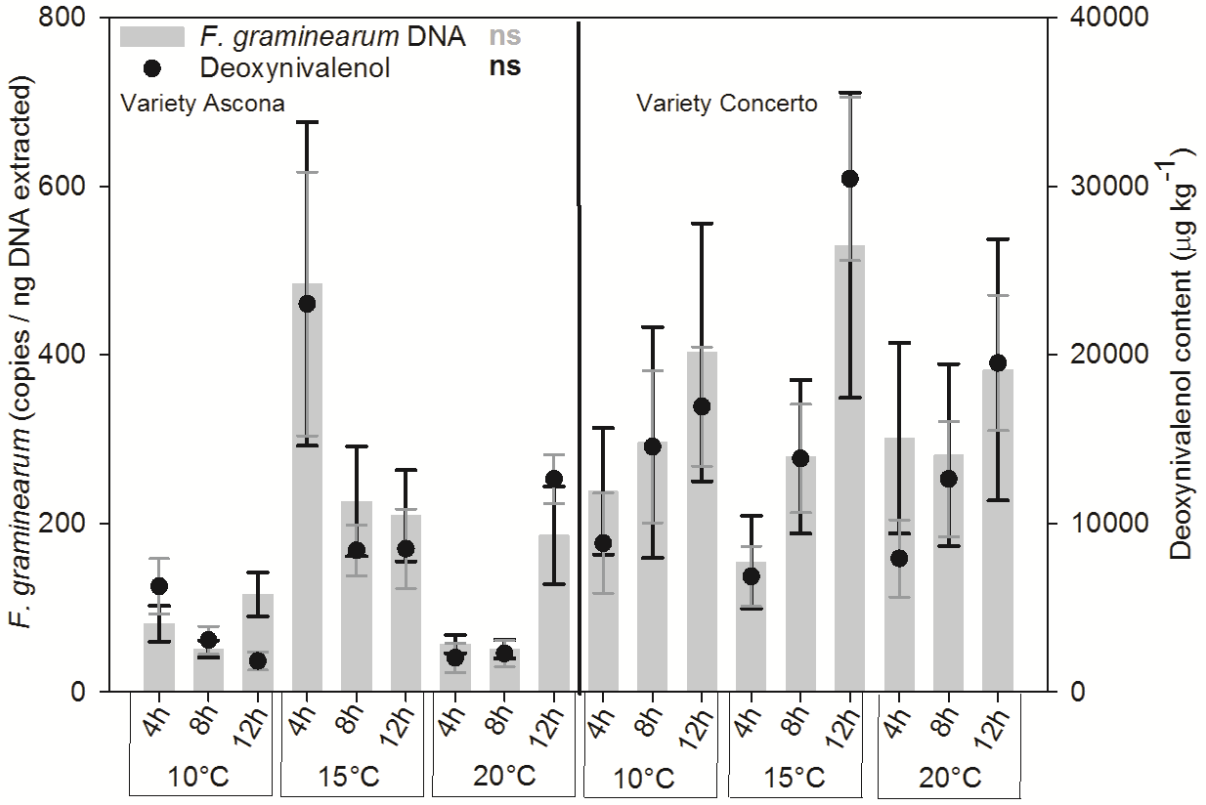

Figure 14: Effect of barley variety, temperature and duration at 99% relative humidity on *Fusarium graminearum* DNA amount (copies/ng DNA extracted) and deoxynivalenol content (µg kg^{-1}) in grains in a climate chamber. Pooled data from two experimental runs. n = 108. Error bars represent the standard error of the mean. ns = not significant.

Weather conditions and spore trapping

During the growth stages DC 25 to 51 (time of straw application until ear emergence), weather conditions in 2015 were characterised by a higher precipitation but a slightly lower mean rH compared with 2016 (Table 19). During DC 51 to 59 (ear emergence) a higher precipitation and rH was observed in 2015 compared with 2016. During anthesis (DC 61 to 69), less precipitation and higher temperature was observed in 2015 compared with 2016 (Table 19). After anthesis, more precipitation was observed in 2016 than in 2015. Generally, temperatures in 2016 were lower than those in 2015.

In 2015, high precipitation (> 10 mm) and temperatures between 11 °C and 16 °C were recorded one week before anthesis, 1.2 mm at DC 61 (beginning of anthesis), no precipitation at DC 65 (full anthesis) and 20 mm at DC 69 (end of anthesis).

Table 19: Mean relative humidity (%), mean temperature (°C), sum of precipitation (mm) during different barley growth stages (DC) measured in 2015 and 2016.

Year	Period	DC*	Relative humidity (%)	Temperature (°C)	Precipitation (mm)
2015	24.03. – 01.05.	25-51	68	9.6	204
2015	02.05. – 06.05.	51-59	84	15.0	69
2015	07.05. – 15.05.	61-69	68	15.8	24
2015	16.05. – 25.06.	71-92	73	16.1	135
2016	17.03. – 03.05.	25-51	75	8.5	110
2016	04.05 – 09.05.	51-59	60	13.4	0.0
2016	10.05. – 16.05.	61-69	79	12.1	91
2016	17.05. – 07.07.	71-92	78	16.5	265

* 25 = main stem and five tillers, 51 = tip of ear just visible, 59 = ear emergence complete, 61 = beginning of anthesis, 69 = anthesis complete, 71 = kernel water ripe, 92 = Grain hard, not dented by thumbnail; according to Zadoks et al (1974).

In 2016, no rain and temperatures between 9 °C and 16 °C were measured at DC 57/59 (end of ear emergence) and at DC 61 and DC 69 (Figure 15). Temperatures above 15 °C were only measured during the first two days of DC 61. During DC 65 (anthesis half way) the highest amount of precipitation was observed, with three days more than 20 mm (Figure 15).

Due to sampling errors, no spore trap data were available in 2015. In 2016, more FG colonies were detected at the end of anthesis (Figure 15). On average, more FG colonies were detected 6 days before anthesis (5.4 colonies per trap per day) than during anthesis (3.4 colonies per trap per day) or three days after anthesis (3.2 colonies per trap per day). Apart from FG, *F. equiseti*

was the second most occurring species detected in the spore traps, with 8 colonies during the entire sampling season. Other *Fusarium* species were only found once including *F. crookwellense*, *F. avenaceum*, *F. proliferatum*, *F. sporotrichioides* and *F. poae.*

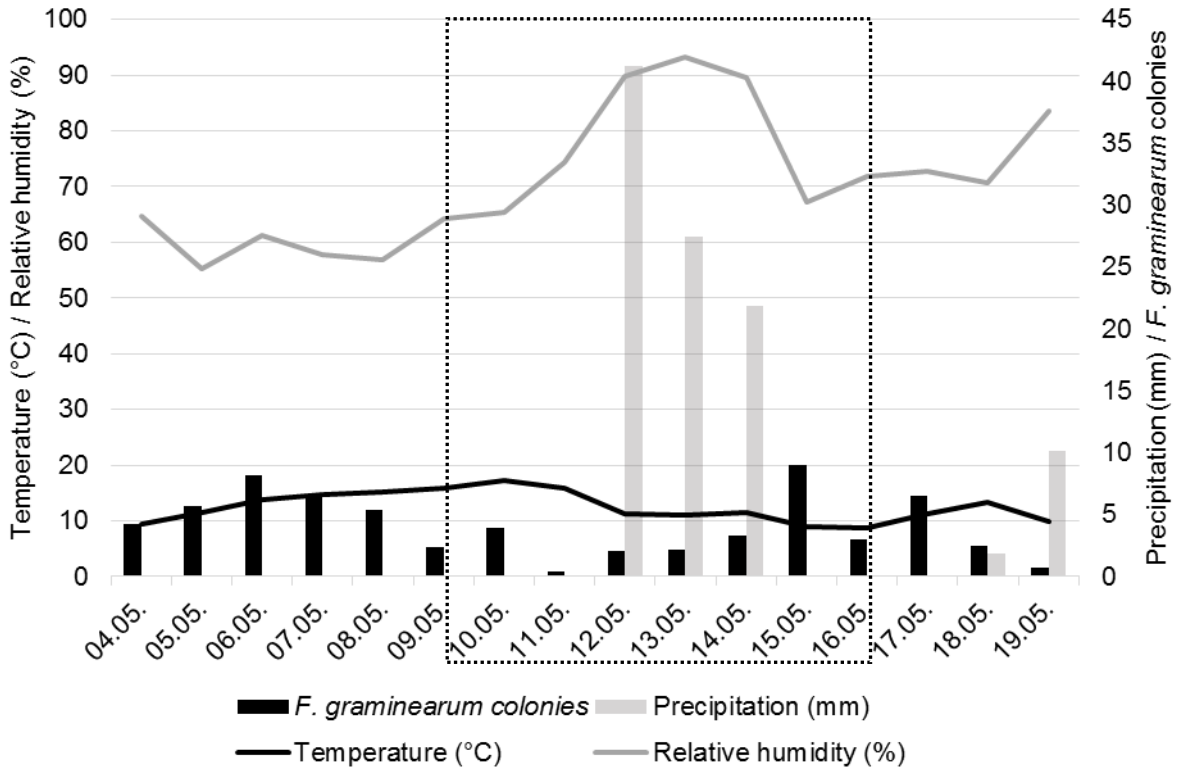

Figure 15: Weather data and detected *Fusarium graminearum* colonies on Pentachloronitrobenzene agar plates between 04.05.2016 and 19.05.2016 (1 day intervals) in a field experiment. Weather data are represented by the sum of precipitation (mm), the mean temperature (°C) and the relative humidity (%). The anthesis period of barley (10.05.2016 – 16.05.2016) is indicated by the dotted black frame. No spore trap data are available for the year 2015.

Field experiment

Disease severity

The disease severity was significantly higher ($p < 0.001$) in 2016 compared with 2015 for both varieties, Meridian and Semper. Averaged over the straw treatments, a significantly higher ($p < 0.001$) severity was observed in grains from the variety Semper compared with the variety Meridian in 2016 (Figure 16). In 2015, a significantly higher disease severity ($p = 0.020$) was observed on grains of the variety Meridian from the freshly inoculated straw plots (2.8%) compared with the overwintered straw plots (0.8%). No further significant differences in disease severity were observed between the three straw inoculation treatments in any of the two years (data not shown).

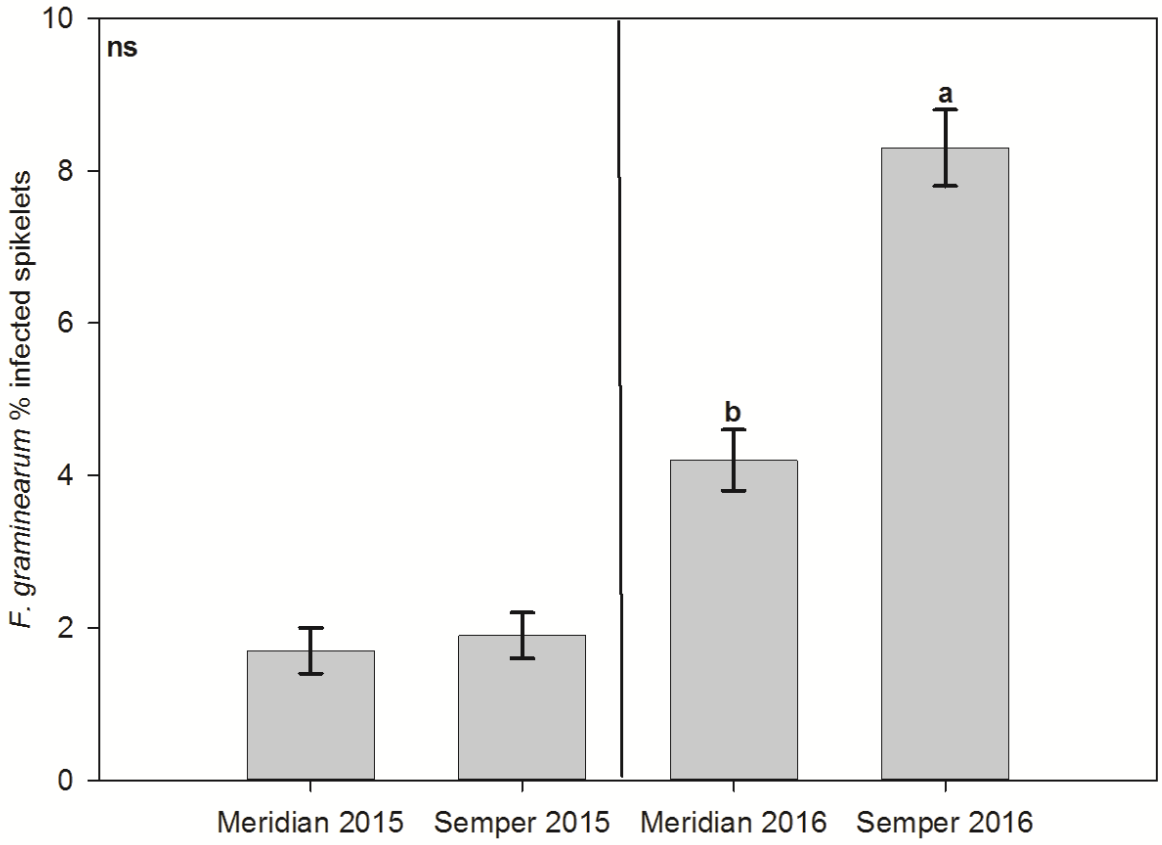

Figure 16: Effect of the barley variety and the year on *Fusarium graminearum* (FG) disease severity (%) based on the number of infected spikelets of the varieties Meridian and Semper in 2015 (n = 24) and 2016 (n = 24) at the first diseases rating in a field experiment. The statistical analysis was conducted for each year separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$; ns = not significant.

Fusarium graminearum incidence

In both years, 2015 and 2016, FG was the dominant *Fusarium* species in the grains, with incidences of 100% and 96%, respectively. In 2015, from all other identified *Fusarium* species, only *F. poae* (0.5%) was detected. In 2016, 2% *F. avenaceum* and 1% *F. poae* were identified. In 2015, averaged over all treatments, the FG incidence in harvested grains from the variety Meridian was significantly higher ($p = 0.043$) compared with the variety Semper (Figure 17). Similar to the disease severity, a strong year effect was found for the FG incidence in grains which was for the variety Meridian significantly ($p = 0.033$) lower in 2015 (13%) compared with 2016 (23%). The same was observed for the variety Semper ($p < 0.001$) with 4% FG in 2015 and 27% in 2016 (Figure 17).

In 2015, a significantly higher FG incidence (Meridian: p = 0.023; Semper: p = 0.038) was observed in grains from the overwintered straw treatment compared with the control treatment (Figure 18).

Deoxynivalenol content

The maximum level for unprocessed barley of 1'250 µg kg^{-1} was exceeded four times in 2015 and never in 2016. In 2015, no DON was detected in grains of the control plots of both varieties. No significant difference was observed between the varieties in any of the years. For Meridian, the average DON contamination was nearly equal in both years with 451 µg kg^{-1} in 2015 and 441 µg kg^{-1} in 2016. Grains from the variety Semper showed a higher mean contamination in 2015 (743 µg kg^{-1}) compared with 2016 (520 µg kg^{-1}) (Figure 17). A significant effect of the straw inoculation treatments on the DON content was only observed in 2015, where grains of the variety Meridian from plots with overwintered straw contained more DON compared with those from the control plots and the freshly inoculated plots (p = 0.002, p = 0.004, respectively); the same effect was observed for the variety Semper (p = 0.002, p = 0.006, respectively) (Figure 18).

A significant correlation between FG incidence and DON contamination was observed in 2015 (r = 0.58; p = 0.003) and 2016 (r = 0.59; p = 0.002). In contrast, no significant correlation was detected between the disease severity and the DON contamination in any of the years. The disease severity correlated only in 2015 with the FG incidence (r = 0.49; p <0.001).

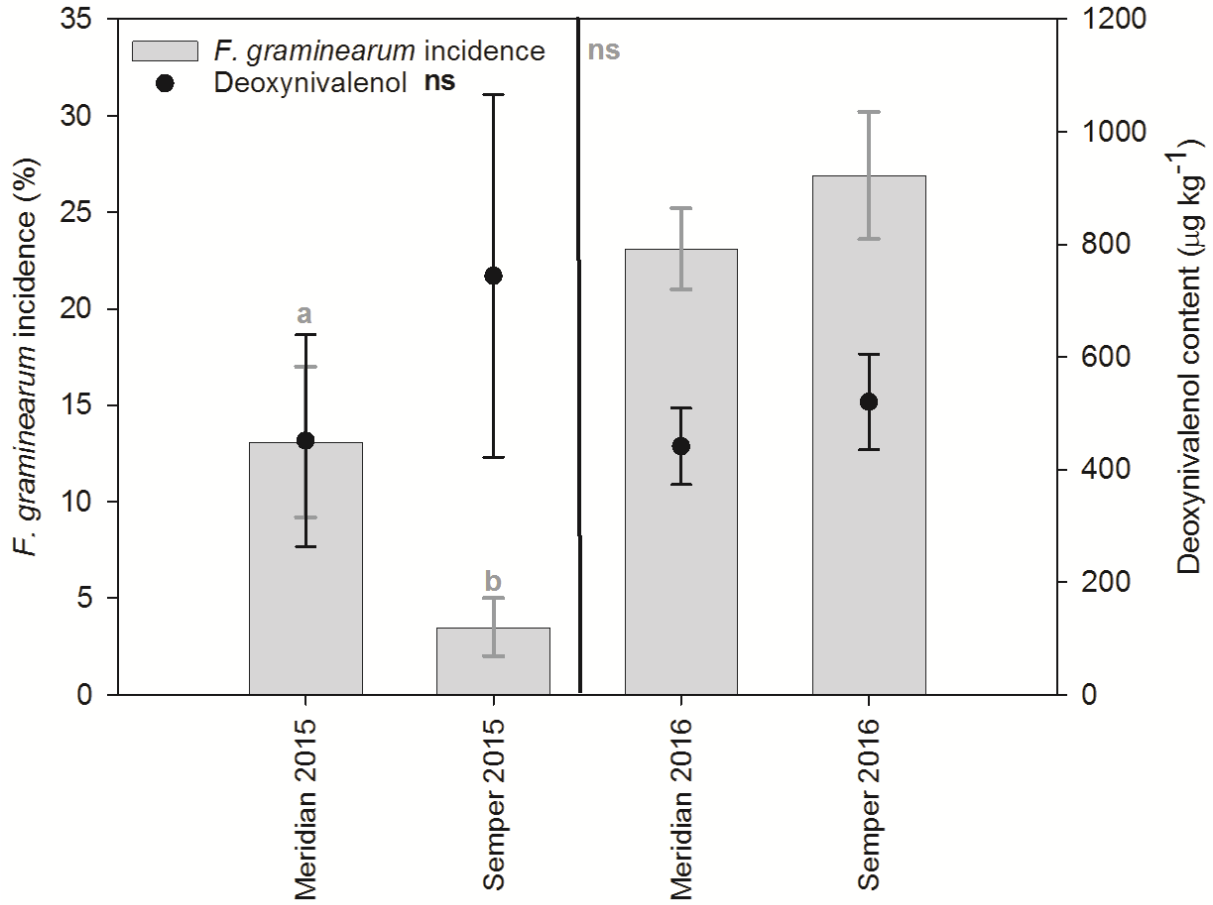

Figure 17: Effect of the barley variety and the year on *Fusarium graminearum* (FG) incidence (%) in grains of the varieties Meridian and Semper in 2015 (n = 24) and 2016 (n = 24) based on a seed health test and on the deoxynivalenol content (µg kg^{-1}) in a field experiment. Grey letters represent significant differences for the FG incidence. The statistical analysis was conducted for each year separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$; ns = not significant.

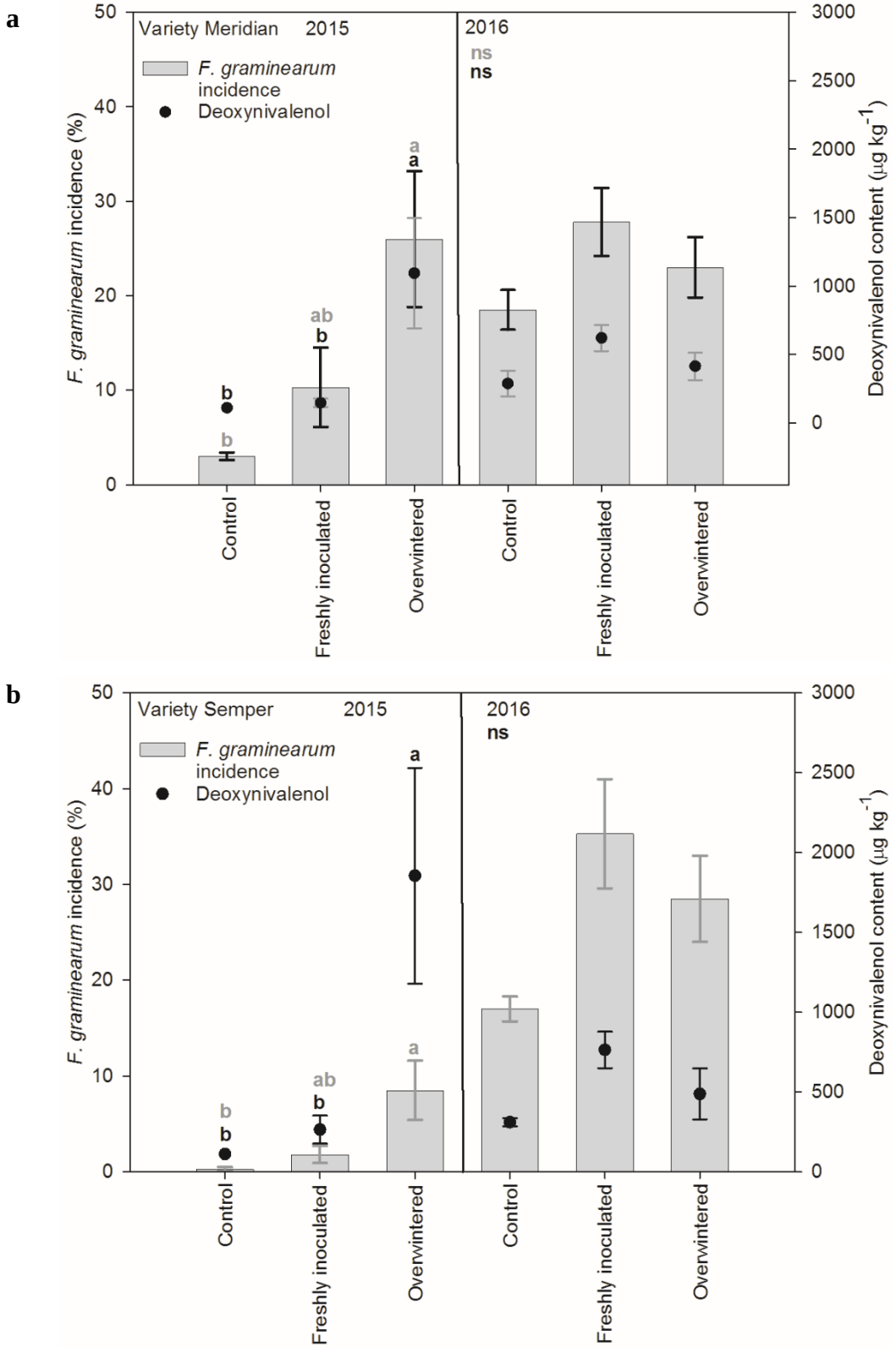

Figure 18: Effect of the straw inoculation treatments and the year on *Fusarium graminearum* (FG) incidence (%) in grains of the varieties **a)** Meridian and **b)** Semper in 2015 (n = 24) and 2016 (n = 24) based on a seed health test and on the deoxynivalenol (DON) content (µg kg^{-1}) in a field experiment. Grey and black letters represent significant differences for the FG incidence and the DON content, respectively. The statistical analysis was done for each year separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$; ns = not significant.

4.5 Discussion

In the current study, we investigated the influence of temperature and humidity duration on FG infections in barley under controlled conditions. Under field conditions, the spore deposition was observed and the effect of differently inoculated straw treatments on FG infection was investigated.

The climate chamber experiments demonstrated that overall, a FG infection of barley is possible under all tested climatic conditions. However, incubation temperatures of 15 °C were shown to be the most favourable in terms of severity and DON contamination. This finding is in contrast to the common knowledge that FG is generally associated with warmer regions of the world and was shown to cause higher infections in wheat at temperatures ≥ 20 °C (Osborne and Stein 2007; Xu et al. 2007a). We assume, that FG strains that infect barley might be more adapted to cooler conditions, due to the earlier anthesis of barley compared with wheat. For the variety Ascona, we observed a higher disease severity and DON contamination in the 10 °C treatments after 4 h 99% rH, compared with the 8 h and 12 h treatments. It is possible that the increased temperature of 20 °C after 4 h of high humidity promoted the growth rate of already germinated spores (Wagacha et al. 2012; Beyer et al. 2004). However, it is not clear yet why this was not observed for the variety Concerto, which in general, showed a higher disease severity and DON contamination.

The prolonged duration of 99% rH revealed only at 20 °C constant results for FG severity, FG DNA amount and DON content in both varieties. However, for variety Concerto the severity increased under all tested temperatures. The variety Concerto was generally more susceptible than Ascona, hence, the differences between the treatments were less distinct.

For the incubation temperature of 20 °C, the longer humidity duration probably enhanced the germination rate and time frame to infect the plant which led to more DON contamination, as shown under controlled conditions in wheat (Xu et al. 2007a; Martínez et al. 2012; Beyer et al. 2005). Hence, at higher temperatures, rainy periods or long periods of mist during barley anthesis can increase the risk of FG infections.

Several studies have shown that FG overwinters on crop residues, which in turn serve as inoculum in the subsequent growing season (Dill-Macky and Jones 2000). Thus, the higher FG incidence in 2015 in the overwintered treatment compared with the freshly inoculated treatment occurred most probably due to a faster colonisation and development of FG on the straw residues. This effect was not observed in 2016, probably due to the more favourable infection conditions, which attenuated the differences between the treatments.

In 2015, the year with less favourable infection conditions, significant difference between the varieties was observed, which might have been due to phenological divergences. Meridian has a shorter growth compared with Semper, so the ears are closer to the ground which facilitates an infection through ejected ascospores in wheat (Maji and Imolehin 2002). In addition, Semper has a lower risk of lodging which is known to enhance the risk of FG infection in barley (Bernhoft et al. 2012).

We assume that the FG infection in 2015 was favoured due to the precipitation before anthesis, washing spores into the flag leaf containing the barley ear with the exposed anthers, which in fact was observed by other authors under field and controlled environment conditions (McCallum and Tekauz 2002; Osborne and Stein 2007). Furthermore, it is possible that barley possesses a prolonged period of susceptibility, since high FG incidences and DON contaminations were observed in a spring barley field experiment when inoculations were conducted as early as DC 51, DC 56 or DC 65 (Stauber et al. 2017). This implies a further difference to wheat, where anthesis is known to be the main susceptible period (Osborne and Stein 2007).

Despite the more favourable infection conditions due to higher precipitation during anthesis (Hooker et al. 2002; Brustolin et al. 2013), only few FG colonies were noted compared with the occurrence before anthesis. This suggests that potentially ejected ascospores were washed down by the higher precipitation or that water covering the perithecia inhibited ejection as assumed by Paulitz (1996). Moreover, during anthesis, temperatures in 2016 were unusually cool. The ejection of ascospores is not only favoured by high humidity but takes place in a range between 10 °C and 30 °C with an optimum near 16 °C (Doohan et al. 2003; Sutton 1982; Paulitz 1996). Indeed, the Swiss forecasting system FusaProg for FHB and DON in wheat calculates main infection and sporulation days with temperatures above 15 °C (Musa et al. 2007). Hence, the temperatures below 15 °C were possibly not favourable for ascospore ejection during anthesis. However, because spore trap data were only available for 2016, the results need to be interpreted with caution.

The higher precipitation occurring after anthesis in 2016 might not only have enhanced the spread of the fungus within the plant but might have also triggered the production of new conidia, since splash dispersal of conidia and ascospores after anthesis under wet conditions was observed in field experiments with wheat (Paul et al. 2004; Manstretta et al. 2015). Furthermore, in a greenhouse and field experiment in Japan, cleistogamous barley varieties were resistant during anthesis but became susceptible afterwards (Yoshida et al. 2007; Yoshida et al. 2008). The barley varieties in our field experiment are both closed flowering and thus,

might have behaved similarly. To test this hypothesis, further climate chamber experiments with different barley varieties inoculated at different growth stages are necessary.

Based on the high disease severity in the field and elevated FG incidences in harvested grains in 2016, we expected a higher DON contamination. However, the measured DON values were nearly equal and in some samples even slightly lower than in 2015. It must be noted that the higher DON content in 2015 was mainly a result of the high contamination of the overwintered straw plots. We assume that the high precipitation at the end of the growing season caused leaching of the water soluble DON, as was observed in field studies artificially spray inoculated with FG in the USA and in Switzerland (Gautam and Dill-Macky 2012; Schenzel et al. 2012).

The close correlations between disease severity, FG DNA amount and DON in the climate chamber demonstrate that a rough estimation of the DON content in barley based on visual assessments is possible under controlled conditions.

Under field conditions however, the disease severity showed only a close correlation with the FG incidences. Only 100 ears of the barley plot were examined for the disease rating, whilst the data of seed health test and DON were based on grains from the entire plot, which provided a higher representative sample. Since in the field, many other factors can influence the appearance of symptoms, we assume that an estimation of the DON content is not possible by visual assessment. Nevertheless, the FG incidences and the DON contents were closely related, which showed that FG was the main DON producer, as observed in other studies with several cereal types (Hietaniemi et al. 2016; Lindblad et al. 2013).

4.6 Conclusion and Outlook

Our results indicate that the optimum temperature for FG infection of barley is 15 °C and is generally enhanced by a prolonged humidity duration, which, in terms of temperature, is different from the warmer infection requirements known for FG in wheat. For the development of a forecasting system for FHB and DON in barley, the potentially longer time frame of susceptibility should be taken in consideration and needs to be verified by more detailed climate chamber and field experiments including several barley varieties. Furthermore, the weather conditions after anthesis until harvest can affect the DON content in the grains and should be investigated in multi-site and multi-year field experiments. Also studies comparing spring and winter barley as well as selected spring and winter wheat varieties should be conducted to observe potential phenological differences such as row type (in case of barley), flowering type and anthesis period on infection with FG and DON contamination. The processing (sterilisation,

mixing and application) of the inoculated straw, as well as the weighing and distribution in the field resulted in a large volume. Hence, for future studies we recommend to use maize stalks.

4.7 Acknowledgements

The authors thank Andreas Kägi for outstanding technical support during the field experiments. We thank Eveline Jenny for her help in conducting the molecular work. Excellent assistance during the sample preparation was provided by Denise Bönisch, Fionna Loeu and Lea Stauber. This work was funded by the Swiss National Science Foundation in the frame of the national research programme 'Healthy Nutrition and Sustainable Food Production' (NRP 69) [grant number 406940_145210].

No potential conflict of interest was reported by the authors.

4.8 Supplementary Material

Table 20: Origin of *Fusarium graminearum* strains, all isolated in Switzerland in 2013.

Strain number	Barley type	Variety	Geographic origin (community/canton)
FG 13170	Winter barley	Landi	Heitenried / Freiburg
FG 13192	Winter barley	Zoom	Känerkinden / Basel-Land
FG 13269	Spring barley	Quench	Cumbel / Grisons

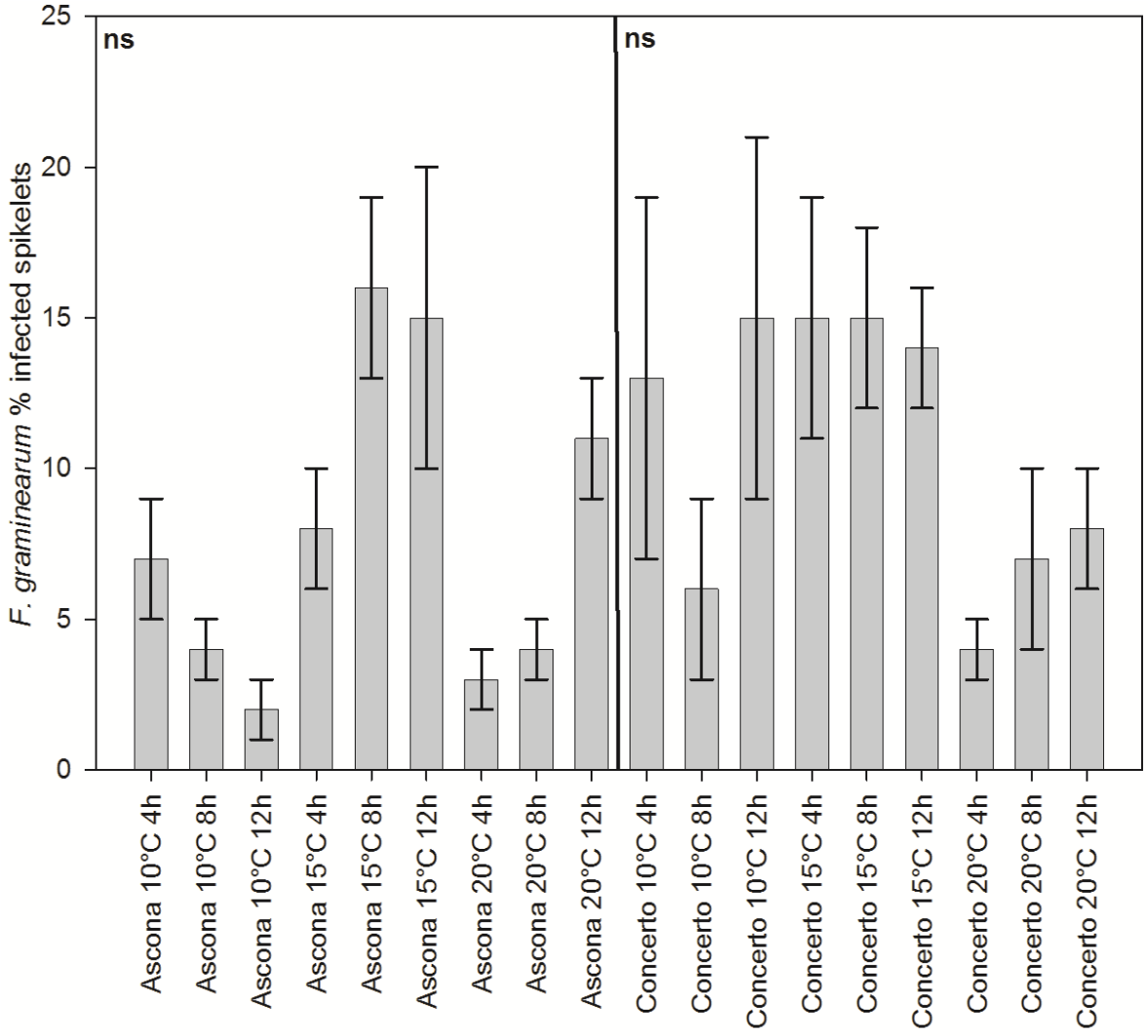

Figure 19: Effect of the barley variety, temperature and duration of 99% relative humidity on *Fusarium graminearum* (FG) disease severity (%) based on the number of infected spikelets after 7 dpi (days post inoculation) in a climate chamber. Pooled data from two experimental runs. n = 108. The statistical analysis was conducted for each variety separately. Error bars represent the standard error of the mean. ns = not significant.

Table 21: Overview of the applied amount of fertilisers, active ingredient of the plant protection agents and control of the respective diseases in field experiments in 2015 and 2016 at the indicated growth stages (DC).

Year	Growth stage (DC)*	Fertiliser (kg ha^{-1})	Active plant protection ingredient	Control of:
2015	DC 00 (sowing) and 18	60% water soluble potassium oxide (300)		
2015	DC 23 and DC 45	25% Mg-ammonium nitrate (75)		
2015	DC 25		metsulfuron-methyl and thifensulfuron-methyl (0.12 kg ha^{-1}) fluroxypyr (1 l ha^{-1})	Monocotyledonous and dicotyledonous weeds
2016	DC 00	60% water soluble potassium oxide (150)		
2016	DC 23, 61 and 75	27% Mg-ammonium nitrate (150)		
2016	DC 35		fluroxypyr (0.75 l ha^{-1}) flupyrsulfuron-methyl (0.02 kg ha^{-1})	Monocotyledonous and dicotyledonous weeds
2016	DC 75		spinosad (0.1 l ha^{-1})	Cereal leaf beetles

* DC 00 = Dry seed, 18 = eight leaves emerged, 23/25 = main stem and three/five tillers, 35 = fifth node detectable, 45 = boots swollen, 61 = beginning of anthesis, 75 = medium milk; according to Zadoks et al. (1974)

5 Epidemiology of *Fusarium langsethiae* and *F. poae* in oats – lower temperatures during anthesis increase mycotoxin accumulation

Submitted to Plant Pathology

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5.1 Abstract

High occurrence of *Fusarium poae* (FP) and *F. langsethiae* (FL) and their mycotoxins nivalenol (NIV) and T-2/HT-2 have been observed in Swiss oats. Early prediction of mycotoxin levels is important for farmers and the cereal industry to minimise the risk of contaminated food and feed. Therefore, climate chamber experiments were conducted to investigate the influence of different temperatures (10 °C, 15 °C, 20 °C) and durations (4 h, 8 h, 12 h) at 99% relative humidity (rH) on the infection of oats with FP and FL. In addition, to discover the most susceptible period of oats, artificial FL infections were conducted at different growth stages. Field experiments were performed to observe the spread of the fungi within the field and to investigate the weather conditions that influence the dispersal. The climate chamber experiments revealed a higher contamination with NIV and T-2/HT-2 in the 10 °C treatments and with a prolonged humidity duration of 12 h 99% rH. Infections of the oat plants at early and full anthesis led to a higher FL infection and T-2/HT-2 accumulation in the grains compared with growth stages before anthesis, which might be due to an increased susceptibility during anthesis. No indication for wind or rain splash transport was observed in the field experiments.

The obtained results, together with influencing cropping factors, can be useful to develop forecasting models to predict a contamination of oats with the mycotoxins NIV and T-2/HT-2.

5.2 Introduction

Fusarium head blight (FHB) is caused by different *Fusarium* species and was extensively studied in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (e.g. Osborne and Stein 2007; refer to chapter 2). Infections lead to yield reductions and contamination with mycotoxins, which threaten human and animal health (Desjardins 2006).

Recently, a higher occurrence of *Fusarium poae* (FP) and *F. langsethiae* (FL) and their mycotoxins nivalenol (NIV) and T-2/HT-2 toxins (T-2/HT-2), respectively, were observed in oats (*Avena sativa* L.) (Hofgaard et al. 2016a; Edwards et al. 2012). These *Fusarium* species do not produce any symptoms in oats and in consequence, healthy looking grains can contain elevated levels of mycotoxins (Imathiu et al. 2009; Stenglein 2009).

To reduce the risk of mycotoxin contaminated food, the European Commission has set indicative levels for the sum of T-2 and HT-2 in 2013 (1'000 µg kg^{-1}) and a tolerable daily intake (TDI) of 0.02 µg kg^{-1} body weight (The European Commission 2013). In addition, an acute reference dose of 0.3 µg kg^{-1} body weight was set in 2017 by the European Food Safety Authority (EFSA) (EFSA 2017). Although, no maximum level has been implemented, the EFSA has set a TDI of 1.2 µg kg^{-1} body weight for NIV which is slightly higher compared with the TDI of deoxynivalenol (DON) (1 µg kg^{-1} body weight) (EFSA 2013b, 2013a).

The toxic effects of NIV and T-2/HT-2 include the inhibition of protein synthesis, reduction of the immune defence and affect the growth in exposed animals (Torp and Langseth 1999; Poapolathep et al. 2002). During oat processing steps (de-hulling, milling and flaking), a removal of these toxins occurs, but is accompanied by an increase in the resulting by-products that are often used as animal fodder (Pettersson et al. 2011).

Epidemiological knowledge about *Fusarium* species is needed to a) understand the disease and ultimately to b) predict mycotoxin levels in harvested grains. This can be an important tool for farmers to reduce an infection with the respective *Fusarium* species and can help to minimise potential food and feed safety problems. Therefore, knowledge about the most influencing weather conditions that affect the infection is essential. The relationship between weather conditions during anthesis and accumulation of mycotoxins in the harvested grains was mainly studied for DON (Hjelkrem et al. 2016; Hooker et al. 2002). The factors temperature and rainfall were shown in numerous studies to affect the infection with *Fusarium graminearum* (FG) and

subsequent toxin accumulation (e.g. Hooker et al. 2002). Until to date, the environmental factors that facilitate FL and FP infection and T-2/HT-2 and NIV production, respectively, remain unclear and no forecasting systems for these *Fusarium* species and toxins are available. The main objectives of the current study were (I) to assess the critical temperature and humidity conditions for a FP or FL infection in oats and (II) to identify the most susceptible growth stage(s) for an FL infection in oats under controlled conditions. Under field conditions, the spread of FP and FL and the influence of weather conditions on the infection in oats were examined and differently treated oat straw residues were used to investigate the overwintering potential of FP and FL.

5.3 Material and Methods

Inoculum preparation

The inoculum preparation of FP and FL for the straw used in the field experiment was done as described in Martin et al. (2017) with the following modifications: colonies were grown on Potato Dextrose Agar (PDA) for one week at 12 h UV-light / 12 h dark at 18 °C. One hundred ml autoclaved V8-medium in a 250 ml Erlenmeyer flask were used and closed with a sterile plug.

The inoculum preparation for the climate chamber experiments were done according to Vogelgsang et al. (2008a) with the following modifications: inoculum production was executed on PDA and each plate was washed with 20 ml 0.0125% Tween® water.

All used *Fusarium* strains were isolated from Swiss oats samples (refer to chapter 3). The geographic origin and the host from which they were derived are shown in Table 22 in the supplementary material (chapter 5.7).

Climate chamber experiments

Two spring oat varieties, Gaillette and Husky were used to study the epidemiology of FP. To ensure a homogenised development of the plants, uncoated seeds were put into germination boxes lined with three wet filter papers (150 x 130 mm; Type 615, Macherey-Nagel, Düren, Germany) and incubated for 36 h at 5 °C in the dark. Subsequently, the boxes were placed into an incubator (RUMED, Rubarth Apparate GmbH, Laatzen, Germany) at 18 °C for 72 h. Three germinated seedlings were planted in pots (ø 15 cm, 13 cm height) in standard soil (Oekohum GmbH, Herrenhof, Switzerland) at a depth of 2-3 cm. Climate chamber conditions were

18 °C / 75% relative humidity (rH) during the day and 13 °C / 85% rH during the night for the first 14 days, then 20 °C / 70% rH (day) and 15 °C / 80% rH (night) until decimal code (DC) 83 (Zadoks et al. 1974). The conditions were then set to 22 °C / 70% rH (day) and 18 °C / 70% rH (night) until harvest. From DC 41 until DC 83, each pot was fertilised once per week with 250 ml Wuxal® P Profi (Manna, Düsseldorf, Germany) (dilution of 1ml/l) per pot.

During anthesis (DC 65), in total three panicles per pot, one per plant, were spray inoculated at a pressure of 2 bar with 30 ml spore suspension adjusted to a concentration of $1*10^6$ spores ml^{-1} containing three different FP strains indicated in Table 22 in the supplementary material (chapter 5.7) to mimic a population. After inoculation, the plants were transferred to a climate chamber to test different temperature and humidity durations at 99% rH. The tested combinations were: 10 °C / 4 h, 10 °C / 8 h, 10 °C / 12 h, 15 °C / 4 h, 15 °C / 8 h, 15 °C / 12 h, 20 °C / 4 h, 20 °C / 8 h and 20 °C / 12 h. A suspension with 0.0125% Tween® water at 20 °C / 12 h served as a control treatment. Immediately after the respective treatments, the plants were placed back into the climate chamber with the aforementioned conditions.

The climate chamber experiment with FL was conducted solely with the oat variety Husky. The temperature and humidity combinations were the same as described for FP. The experiment was augmented by infections at different development stages, because it was still unknown whether FL is able to infect before anthesis. The plants were either inoculated at the beginning of ear emergence (DC 51), half of ear emergence (DC 55), early anthesis (DC 61) or full anthesis (DC 65).

In both experiments, three repetitions of each temperature and humidity duration combination were conducted. Both experiments were repeated twice. For Gaillette, the first repetition of the experiment was excluded from the statistical analysis due to technical problems with the climate chamber programme during the anthesis and incubation period. In addition, one replicate of the treatment 10 °C / 12 h 99% rH from the second experimental repetition of variety Gaillette was lost, due to a milling error.

Field experiments

The field experiments were conducted at the Agroscope research institute in Zurich, Switzerland (N 047° 24 430; E 008° 30 597) over two years 2015 and 2016. The previous crop in both years was potato.

Straw inoculation

The treatments (plot size 3 m x 9 m) comprised the three straw inoculation methods: I: autoclaved straw, II: freshly inoculated straw and III: overwintered straw. For the "overwintered straw" treatment, oat straw from field experiments artificially inoculated during anthesis in the previous year with FP or FL was taken and stored at 5 °C in the dark. For the control treatment, "autoclaved straw" and the freshly inoculated treatment, barley straw harvested in 2014 was autoclaved for 30 min at 121 °C. For the treatment, "freshly inoculated", the straw was inoculated in wooden boxes by stacking one layer of straw and subsequently spraying a spore suspension ($1*10^6$ *Fusarium* spores ml^{-1}) of FP or FL before adding the next layer of straw. This was repeated for several times. The same procedure was done for the control treatment, and the "overwintered straw" treatment using only Tween® water. The applications were done using a backpack sprayer (Flox 10, Birchmeier, Stetten, Switzerland). Twenty four kilogram straw per treatment were sprayed with twelve litres of the spore suspension or Tween® water. The wooden boxes were placed into climate chambers at 20 °C, 99% rH for two to three days. Once per day, the straw was mixed by hand, to ensure a homogenised infection.
Seedbed preparation was done by ploughing (September 2014; October 2015) followed by grubber (November 2014, December 2015) and subsequently harrowing treatments (March 2015/2016). Sowing of the two spring oats varieties Husky and Zorro was done in March of the respective year. The varieties were chosen according to the recommended Swiss variety list. The experiments consisted of a randomized complete block design with four replicated blocks. For FP, each treatment was employed with the varieties Zorro and Husky. For FL, only the variety Husky and the control and freshly inoculated treatment were tested in 2015 since no overwintered straw for the third treatment and no additional field area for a second variety was available. Next to the plots where the straw was applied, a side plot with either Husky or Zorro of the same dimension was sown to observe potential spread into border plots. Each plot was surrounded by triticale plots of 3 m x 12 m to minimise the risk of cross contamination. The straw was applied to the respective plots at DC 26 in 2015 and at DC 29 in 2016 due to heavy precipitation during DC 26 in 2016. For each plot, 2.5 kg straw was applied. All plots were fertilised twice (DC 22/23 and 45/47) with 120 kg ha^{-1} 24% Mg-ammonium nitrate. In 2015, the insecticide Karate with Zeon Technology® (Syngenta, Dielsdorf, Switzerland) was applied once to control aphids and cereal leaf beetles. In 2016, the herbicides Concert® SX and Starane® 180 (Stähler, Zofingen, Switzerland) and the insecticide Talstar® (Stähler) were used to control monocotyledonous and dicotyledonous weeds and straw flies, respectively. No growth regulators were applied in any of the years. All plots were harvested

with a plot combine harvester at the end of July 2015 and beginning of August 2016, respectively. Weather data were obtained during the entire cropping season using a nearby weather station operated by the Federal Office of Meteorology and Climatology (MeteoSwiss).

Spore traps

Observation of the spore dispersal during anthesis was done by the aid of spore traps. These consisted of an iron rod that was bent at a right angle at the upper end. At this angle, two holes were drilled and a wooden board about 30 cm long and 10 cm wide was screwed onto the iron rod. An aluminium dish (15 x 11 x 4.5 cm, type 150550, Company Pacovis AG, Stetten, Switzerland) was placed in a vertical position on the board. At the upper end of the aluminium dish, a second one of the same type was attached in a horizontal position as shown in Figure 20. A Petri dish (∅ 9 cm) with a *Fusarium* selective agar medium (containing pentachloronitro-benzene (PCNB), Sigma-Aldrich®, Buchs, Switzerland), was placed under this construction, and was thus protected from rain, direct sunlight and rapid drying out. In addition, two small nails were placed at a distance of nine cm from the vertical aluminium dish in order to fix the Petri dish onto the board.

The spore traps were placed on the height of the oat heads in northerly direction in order to protect the agar plates by the vertical aluminium dish from higher temperatures and irradiation at noon. For each plot, one spore trap was placed during the anthesis period and agar plates were exchanged every 24 h and subsequently incubated at 12 h UV / 12 h darkness at 18 °C for 6 days. To identify the different *Fusarium* species, colonies were transferred onto PDA and SNA (Spezieller nährstoffarmer Agar (Leslie and Summerell 2006)) and incubated at the above mentioned conditions. The different *Fusarium* species were identified based on their morphology, according to the manual by Leslie and Summerell (2006).

Fusarium species incidence

Incidence of *Fusarium* species in oat grains from field experiments were determined with the seed health test method as described in Vogelgsang et al. (2008b) and identified as described above.

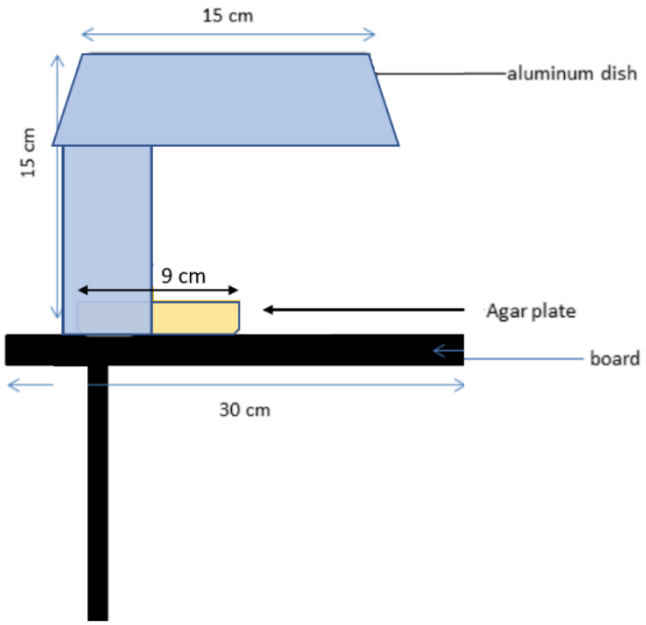

Figure 20: Schematic construction of the spore trap used in the field experiments.

Quantification of *Fusarium* species by quantitative PCR

DNA extraction was done as described in chapter 3.3. The DNA concentration in the milled grain samples from plants grown in the climate chamber and milled grain samples from the field experiments was determined by measuring the emission of the fluorescent Hoechst 33258 (Fluorescent DNA Quantitation Kit; BioRad, Cressier, Switzerland) bound to double stranded DNA based on the emitted fluorescence of a known concentration of serially diluted DNA standard (calf thymus DNA (BioRad)) after excitation at 460 nm in a Cary Eclipse UV/Visible Spectrophotometer (Agilent technologies, Basel, Switzerland).

The quantitative PCR (qPCR) for FP was done as described in chapter 3.3 whereas the FL qPCR was done according to Edwards et al. (2012) with the respective modifications explained in chapter 3.3. The lowest standard (SQ 20) was set as the limit of detection (LOD) and sample values below the LOD were replaced by a constant value of LOD/2.

Mycotoxin measurement in harvested grains

Extraction of mycotoxins was done as described in chapter 2.3 with the following modifications. Five grams milled sample were weighed into a 50 ml glass bottle and extracted with 20 ml solvent mixture (acetonitrile/acetone/Milli-Q® water (50:25:25)). Due to reduced sample amounts for the climate chamber experiments, only 2 g flour were extracted with 8 ml solvent. Filtered extract was cleaned additionally by drawing 2 ml extract over 0.60 mg of

Alox:Celite (50:50; Alox activated 6 h by 400 °C) in 3 ml SPE tubes by the aid of vacuum, instead of 1 ml extract with 0.15 mg. Cartridge holder, valve and needle of the SPE-box (Visiprep Vacuum Manifold, Supelco, Bellefonte, PA, USA) were washed between each sample. Finally, 0.2 ml extract was transferred with a Gastight Syringe (Hamilton, Bonaduz, Switzerland) into a 1.5 ml crimp vial (BGB, Böckten, Switzerland), reduced to complete dryness by a gentle airstream at maximal 50 °C, resuspended in 1 ml of water : methanol (90:10), crimped and vortexed 10 seconds.

Separation and detection was performed on an Agilent 1260 LC-System coupled to an Agilent 6470 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA)). All samples were analysed for the following A and B trichothecenes: NIV, DON, fusarenon-X, acetyldeoxynivalenol, neosolaniol (NEO), diacetoxyscirpenol, T-2, HT-2 and for zearalenone (analytical grade, r-biopharm, Darmstadt, Germany). Separation of trichothecenes was performed at 40 °C on a Zorbax Eclipse Plus 18 column (1.8 µm, 2.1 x 50 mm; Agilent), equipped with a guard column (SecurityGuardTM Cartridge C18 4 x 2.0 mm, Phenomenex Inc., Torrance, CA, USA). Injection volume was set to 10 µl with a mobile phase flow rate of 0.30 ml min^{-1}. Eluent A consists of Milli-Q® water/methanol (95/5 v/v) and eluent B of Milli-Q® water/methanol (5/95 v/v). To enhance the ionization process in the interface, both eluents were fortified with ammoniumacetate (LC-MS grade, Fluka, Buchs, Switzerland) to a final concentration of 5 Millimolar. Gradient parameters of the LC-System and interface parameters of the mass spectrometer are shown in Table 23 and Table 24 in the supplementary material (chapter 5.7), respectively.

The T-2/HT-2 toxins concentration of the milled grains from the FL climate chamber experiment was measured using a competitive Enzyme Linked Immunoabsorbent Assay (ELISA), Ridascreen® T-2/HT-2 Toxin kit (R-Biopharm AG, Darmstadt, Germany). Sample preparation and ELISA assay was conducted according to manufacturer instructions. Samples extinctions were measured using a spectrometer (Tecan Sunrise™, Männedorf, Switzerland) and the toxin concentrations were estimated via a standard curve of known T-2/HT-2 concentrations by Ridasoft Win 1.84 software (R-Biopharm AG). The LOD was 16 µg kg^{-1} and values below the LOD were replaced by a constant value of 8 µg kg^{-1}.

Statistical analysis

For statistical analysis, the software R Version 3.3.2 and R Studio Version 1.0.136 with the packages agricolae, nlme and lsmeans was used (R Core Team 2015; R Studio Team 2015; de

Mendiburu 2015; Pinheiro et al. 2016; Lenth 2016). Data from all experiments were verified graphically for homogeneity of variances and normality of residuals. Mycotoxin and qPCR data were log transformed and seed health test data were arcsine square root transformed to meet the requirements for the post-hoc and correlation tests.

To investigate significant influencing factors, a linear mixed effects model was created followed by an analysis of variance (ANOVA). For the climate chamber experiment, the effects of temperature, humidity duration, growth stage, and variety were set as fixed factors, whereas experiment repetition and block were set as random factors. For the field experiments, year, straw treatment and variety were set as fixed factors and block as random factor. Obtained significant factors were then examined using the Tukey method for pairwise comparison of least-square means ($\alpha = 0.05$).

Using the transformed data (see above), Pearson correlation was calculated to determine the relationship between qPCR and mycotoxin data. For figures the software SigmaPlot© Version 13.0.0.83 (Systat Software, Inc.), Microsoft® Excel 2013 and Microsoft® PowerPoint 2013 with untransformed data was used.

5.4 Results

Climate chamber experiment with *Fusarium poae*

The toxin NIV was detected in 80 out of the 81 milled grain samples of the inoculated plants. In 76 out of 81 samples of the inoculated plants, the FP DNA amount was above the LOD. Neither NIV nor FP DNA was determined in plants from the control treatments.

The ANOVA results revealed a significant effect of the factors temperature ($p < 0.001$) and variety ($p = 0.002$) on the FP DNA amount, as well as for NIV (temperature, $p < 0.001$; variety, $p = 0.024$).

Averaged over the humidity durations, inoculations of the variety Husky at 10 °C led to significantly higher FP DNA amounts (56 copies/ng extracted DNA) in oat grains compared with the inoculations at 15 °C (7 copies/ng extracted DNA; $p < 0.001$) and 20 °C (8 copies/ng extracted DNA; $p = 0.004$) (Figure 21).

Averaged over the humidity duration, the NIV content was significantly higher ($p = 0.013$) in grains from the 10 °C treatments (175 µg kg^{-1}) compared with the 15 °C treatments (68 µg kg^{-1}) for variety Husky. The same trend was observed for variety Gaillette, showing a higher

FP DNA amount and NIV content in milled grains from plants inoculated at 10 °C compared with grains from plants inoculated at 15 °C or 20 °C (Figure 21).

Inoculation conditions at 10 °C / 12 h 99% rH revealed the highest mean FP DNA amounts and highest mean NIV concentrations for variety Husky (115 FP copies/ng extracted DNA; 243 µg kg^{-1}) and for variety Gaillette (163 FP copies/ng extracted DNA; 465 µg kg^{-1}) (Figure 25 in the supplementary material (chapter 5.7)). No significant effect of the humidity duration was observed, but prolonged humidity durations revealed a higher FP DNA amount and NIV contamination in grains inoculated at 10 °C (Figure 25 in the supplementary material (chapter 5.7)). A strong positive correlation between FP DNA amount and NIV contamination was observed for both the variety Husky ($r = 0.80$, $p < 0.001$) and the variety Gaillette ($r = 0.79$, $p < 0.001$).

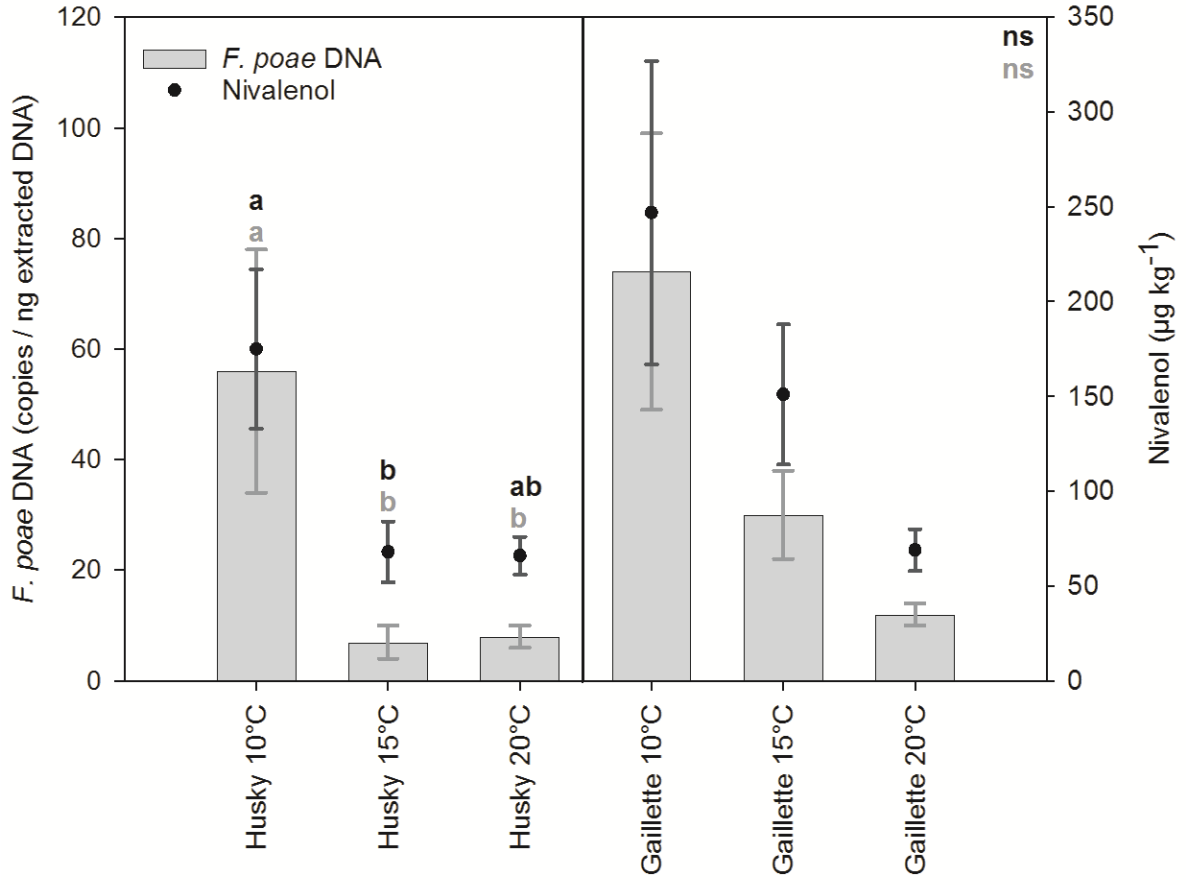

Figure 21: Effect of temperature and variety on *Fusarium poae* (FP) DNA amount (copies/ng extracted DNA) and nivalenol (NIV) content (µg kg^{-1}) in a climate chamber experiment. Pooled data from two experimental runs. n = 81. Light grey and black letters represent significant differences for the FP DNA amount and for NIV, respectively. Analysis were conducted for each variety separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$.

Climate chamber experiment with *Fusarium langsethiae*

In 138 out of the 216 milled grain samples, T-2/HT-2 was detected. Of these 138 samples, 29 were inoculated at DC 51 and 25 at DC 55, whereas grains from plants inoculated at DC 61 and DC 65, revealed 37 and 47 samples above the LOD, respectively. Roughly one third of the samples (71) were above the LOD for FL DNA. In four grain samples out of 54 (7%) inoculated at DC 51 and in six samples out of 54 (11%) inoculated at DC 55, FL DNA was detected. Inoculation at DC 61 and DC 65 revealed 29 (54%) and 32 (59%) samples each out of 54 samples above the LOD, respectively (Figure 22). No FL DNA and T-2/HT-2 was measured in any of the control samples.

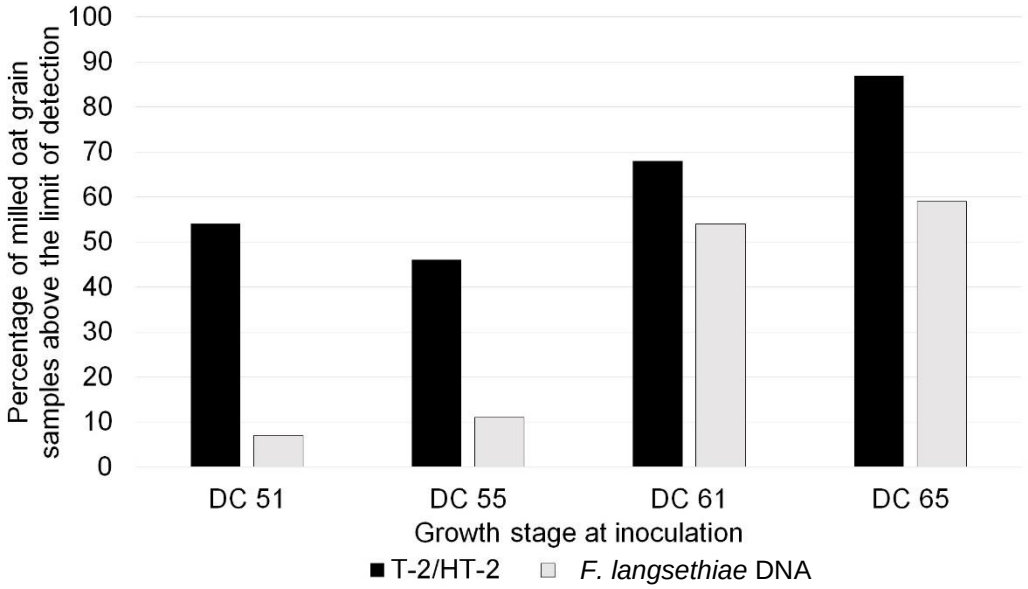

Figure 22: Percentage of samples above the LOD for T-2/HT-2 and for *Fusarium langsethiae* DNA in milled oat grain samples from a climate chamber experiment. Pooled data from two experimental runs (n = 216).

The ANOVA revealed temperature, growth stage and the respective interaction as significant influencing factors (all $p < 0.001$) for the FL DNA amount. For the T-2/HT-2 content, temperature ($p = 0.005$), growth stage and the interaction (both $p < 0.001$) were detected as significant influencing factors. Averaged over humidity duration and growth stage, grains from plants inoculated at 10 °C resulted in significantly higher FL DNA amounts (7.1 FL copies/ng extracted DNA) compared with inoculations at 20 °C (1.7 FL copies/ng extracted DNA; $p < 0.001$) and at 15 °C (2.6 copies/ng extracted DNA; $p = 0.008$) (Figure 23). For the T-2/HT-2 content, this difference was only significant ($p = 0.004$) between the 10 °C treatments (107 µg kg^{-1}) and the 20 °C treatments (32 µg kg^{-1}) (Figure 23).

Averaged over temperature and humidity duration, grains from plants inoculated during the growth stages DC 51 and DC 55 showed significantly lower ($p < 0.001$) FL DNA amounts (0.5 FL copies/ng extracted DNA; 0.6 FL copies/ng extracted DNA) and T-2/HT-2 contents (18 µg kg^{-1}; 44 µg kg^{-1}) compared with grains from plants inoculated during DC 61 and DC 65 (8 FL copies/ng extracted DNA; 69 µg kg^{-1} and 6 FL copies/ng extracted DNA, 143 µg kg^{-1}, respectively) (Figure 23). Within the respective growth stages, temperature and humidity duration had no significant effect on FL DNA amount and T-2/HT-2 content.

However, the highest values of T-2/HT-2 and FL DNA amount were detected in the 10 °C / 12 h 99% rH treatment inoculated during DC 65 (669 µg kg^{-1}; 27 FL copies/ng extracted DNA) whereas the lowest value of T-2/HT-2 was detected in 20 °C / 12 h 99% rH treatment inoculated during DC 55. The T-2/HT-2 data showed a significant positive correlation with the FL DNA amount ($r = 0.74$, $p < 0.001$).

Weather conditions during field experiments

During the growth stages DC 29 to 51 (application of straw until booting), weather conditions in 2016 were characterised by less precipitation and sunshine duration, but a slightly higher mean rH compared with 2015. During DC 51 to 59 (ear emergence) and after flowering until harvest (DC 71 to 89), higher precipitation and rH as well as less sunshine duration were observed in 2016 compared with 2015. During anthesis (DC 61 to 69), less precipitation and a higher sunshine duration was observed in 2016, but no difference of the rH was observed compared with 2015 (Table 25 in the supplementary material (chapter 5.7)). In 2015, no precipitation was recorded during five days within the full anthesis period, whereas in 2016 the highest amount of precipitation (15.3 mm) was recorded at mid anthesis (DC 65). The mean temperatures in the respective growth stage periods did not differ between the years (Table 25 in the supplementary material (chapter 5.7)).

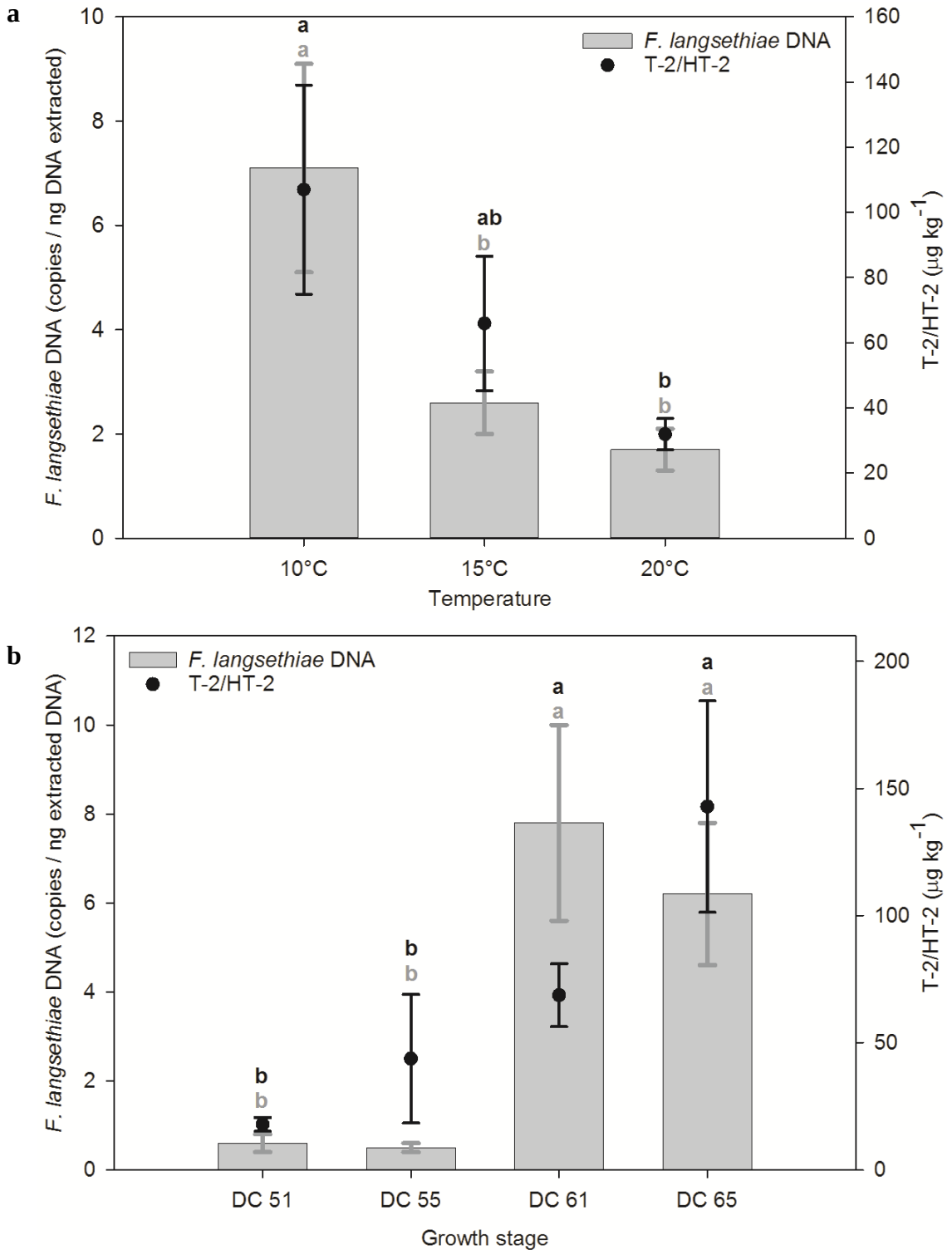

Figure 23: Effect of **a)** temperature during incubation and **b)** growth stage at inoculation (DC 51 = tip of ear just visible; DC 55 = rar half emerged; DC 61 = beginning of anthesis; DC 65 = anthesis half way) on *Fusarium langsethiae* (FL) DNA amount (copies/ng extracted DNA) and T-2/HT-2 contamination (µg kg^{-1}) in milled oat grains from plants grown in a climate chamber experiment. Pooled data from two experimental runs. n = 216. Light grey letters represent significant differences for the FL DNA amount, black letters represent significant differences for T-2/HT-2. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$.

Field experiment with *Fusarium poae*

The factors variety and year showed a significant influence (both $p < 0.001$) for the FP DNA amount and the mycotoxins NIV, DON and T-2/HT-2. In all oat grain samples from 2015 and 2016, NIV and FP DNA were detected. No maximum or indicative legislative value for DON or T-2/HT-2 was exceeded in any of the years.

In both years, averaged over the treatments, the grains of variety Zorro had significantly higher ($p < 0.001$) FP DNA amounts and T-2/HT-2 contents ($p = 0.024$) compared with grains of Husky. In contrast, grains of variety Husky had significantly higher DON contents ($p = 0.025$) (Figure 24).

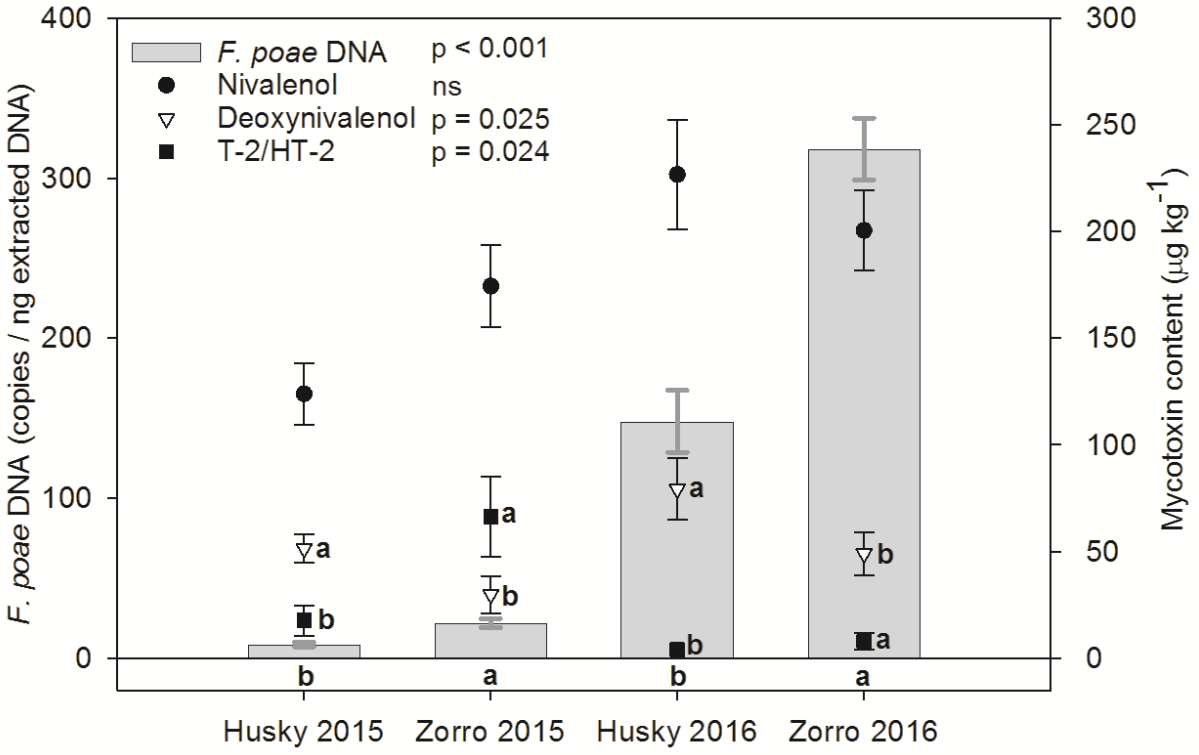

Figure 24: Mean amount of *Fusarium poae* (FP) DNA (copies/ng extracted DNA), mean nivalenol content, mean deoxynivalenol (DON) content and mean T-2/HT-2 content in grains of the oat varieties Husky and Zorro in 2015 (n = 24) and 2016 (n = 24) field experiments. Letters below the bars represent significant differences for the FP DNA amount, letters next to the white triangle represent significant differences for DON and letters next to the black squares represent significant differences for T-2/HT-2. The analysis were conducted for each variety separately. Error bars represent the standard error of the mean, means with the same letters are not significantly different according to a Tukey test at $\alpha = 0.05$; ns = not significant.

Significantly higher NIV contaminations ($p = 0.004$) and FP DNA amounts ($p < 0.001$) were observed in 2016 (214 µg kg^{-1}; 233 copies/ng extracted DNA) compared with 2015 (149 µg kg^{-1}; 149 copies/ng extracted DNA) averaged over all plots. The average contamination with T-2/HT-2 was significantly higher ($p = 0.003$) in 2015 (42 µg kg^{-1}) compared with 2016 (6 µg kg^{-1}). In addition, a higher FG incidence was observed in 2016 compared with 2015 (Table 26 in the supplementary material (chapter 5.7)).

No significant differences in NIV and FP DNA were detected between the three straw treatments. However, significantly higher DON contents ($p = 0.035$) were measured in 2016 grains of the variety Husky in plots with overwintered straw (68 µg kg^{-1}) compared with grains from the freshly inoculated straw plots (32 µg kg^{-1}).

To investigate whether FP is able to spread to neighbouring plots and to test the influence of the main wind direction, grains from the oat border plots were also analysed for their content of mycotoxins and FP DNA. Overall, a lower amount of FP DNA (average of 94 copies/ng extracted DNA) and NIV contamination (153 µg kg^{-1}) was detected in grains from the oat border plots compared with those from the plots where the straw was directly applied (124 copies/ng extracted DNA; 181 µg kg^{-1}). No effect of the main wind direction was observed in terms of inoculum dispersal. The spore traps employed during anthesis contained only small numbers of FP colonies in both years: In 2015 and 2016, FP was detected during three and one day/s, respectively, but only very few colonies (one to four per day) were detected. In fact, FG was the most identified species in both years and a higher occurrence of *F. avenaceum* (FA) was observed in 2015 (data not shown).

Field experiment with *Fusarium langsethiae*

In 2015 and 2016, NIV was detected in all oat grain samples. A contamination with NEO was detected in all samples in 2015 (average of 182 µg kg^{-1}), but none in 2016. No maximum or indicative legislative value for DON or T-2/HT-2, respectively, was exceeded in any of the years.

The straw treatments did not result in significant differences for any mycotoxin or the FL DNA amount in the oat grain samples. The only trend observed in 2016 was a higher DON content in oat grains from both varieties of the overwintered inoculum treatment compared with the other straw treatments (data not shown). Averaged over all straw treatments, the comparison between the two varieties in 2016 showed a significantly higher amount of FL DNA ($p = 0.002$)

in oat grains of the variety Zorro (1.7 copies/ng extracted DNA) compared with Husky (0.5 copies/ng extracted DNA).

A statistical analysis over two years was only possible for variety Husky and the freshly inoculated and the control treatments: Averaged over the treatments, significantly higher FL DNA amount ($p = 0.001$) and T-2/HT-2 contamination ($p < 0.001$) was observed in 2015 (5.9 copies/ng extracted DNA; 59 µg kg^{-1}) compared with 2016 (0.7 copies/ng extracted DNA; 3 µg kg^{-1}). In contrast, in 2016, a significantly higher mean amount of NIV (286 µg kg^{-1}; $p = 0.005$) and DON (26 µg kg^{-1}; $p = 0.018$) was observed compared with 2015 (124 µg kg^{-1}; ns). A higher FG and FP incidence was observed in 2016 compared with 2015 (Table 26 in the supplementary material (chapter 5.7)).

In 2015, significantly ($p = 0.048$) more FL DNA (12.2 copies/ng extracted DNA) was detected in oat grains from the oat border plots compared with plots where straw was applied (5.9 copies/ng extracted DNA). This effect was not observed in 2016 or for NIV and T-2/HT-2 during the two years. In both years, no FL colonies but FG and FA colonies were detected in the spore trap plates.

5.5 Discussion

In the current study, we investigated the influence of temperature and humidity duration on FP and FL infections under controlled conditions. Additionally, the most susceptible growth stage for a FL infection was assessed and under field conditions, the spread and spore dispersal of FP and FL was monitored.

The climate chamber experiments demonstrated that temperatures of 10 °C were more favourable for FP and FL infections of oats. Similar to our results, an *in vitro* experiment revealed a stronger coleoptile growth reduction of FP infected wheat at 10 °C and 15 °C compared to 20 °C and 25 °C, which implies a stronger infection potential of FP under cooler conditions (Brennan et al. 2003). Still, a direct comparison with our results is difficult, because two different syndromes were evaluated.

We assume that FP and FL isolates differ in their adaptation to climatic conditions, because in other studies FP incidence, NIV content and FL infection increased with rising temperatures (Xu et al. 2007a; Opoku et al. 2013). However, these differences may arise, because Xu et al. (2007a) studied wheat plants and Opoku et al. (2013) observed the increase under field conditions, which might be more influenced by other factors and they sampled plant material at the different growth stages, while we analysed harvested grains. Plant resistance is lower

under colder conditions (reviewed in Suzuki et al. 2014), hence we assume that the transfer of the plants from warm conditions (20 °C) to the cold conditions (10 °C) during the infection period could have led to a higher FP and FL susceptibility.

A prolonged duration of 12 h 99% rH resulted in a higher FP DNA amount and NIV content in the harvested grains, which was also observed under controlled conditions in wheat (Xu et al. 2007a). In a study by Divon et al. (2012), bagging oat shoots for six days after spray inoculation resulted in a successful FL infection. However, our results indicate, that a duration of 4 h 99% rH is sufficient for a successful FP and FL infection. Hence, rain and high humidity in combination with low temperatures during anthesis can increase the risk of a NIV or T-2/HT-2 contamination in oats due to a higher germination rate of FP or FL conidia.

The results from the climate chamber experiment with FL imply that an infection at beginning or mid anthesis leads to the highest amounts of FL DNA and T-2/HT-2, particular at temperatures of 10 °C. These conditions do not frequently occur in Switzerland, but may result in a high contamination in northern areas of Europe or in high altitudes. Anthesis is generally thought to be the most susceptible growth stage for a *Fusarium* infection in cereals and a field experiment with oats artificially inoculated with FG during anthesis revealed a higher kernel infection and DON content compared with infection after anthesis (Tekle et al. 2012). Similar to our results, a spray inoculation at anthesis or early dough stage under greenhouse conditions resulted in the highest amount of FL DNA, whereas boot injection before anthesis resulted in low fungal DNA amounts (Divon et al. 2012). In contrast to our study, a higher FL biomass was noticed during head emergence but before anthesis in a field survey in the UK (Opoku et al. 2013). Based on our results, we assume that an infection with FL before anthesis is not important for a high T-2/HT-2 contamination. Since we only tested one oat variety, further research with different oat varieties will be needed to verify whether a variety effect in terms of flowering time or type (open or closed flowering) exists. The strong correlation between FL DNA and T-2/HT-2 implies that FL is a relevant T-2/HT-2 producer in Switzerland as shown in other European countries (Hofgaard et al. 2016a; Edwards et al. 2012).

The reasons for the absence of significant differences between the straw treatments in the FP and FL field experiments remain unclear. Hence, it is still unknown whether FP and FL can overwinter on crop residues on the soil surface and thus might serve as primary inoculum. Although we observed low amounts of FP conidia and no FL conidia in the spore traps, it might be possible that some FP and FL conidia spread within the different treatments, because the border plots were too small to reduce the cross contamination or because conidia were introduced from outside to the experimental field transported by wind. We consider that the

mycelium of faster germinating ascospores or conidia of FG and FA overgrew present FP conidia in the petri dishes as was shown in a co-infection experiment (Xu et al. 2007a). This might hold also true for the rather slow growing FL (Torp and Nirenberg 2004).

We suppose that the main dispersal of conidia did not occur during anthesis, since no FL was detected in the spore traps. In a field trial with naturally contaminated straw in Norway, low amounts of FL DNA were detected in the spore traps and only during late time points, starting from four weeks after heading (Hofgaard et al. 2016b). This implies that spore dispersal of FL probably occurs later in the growing season. Thus, further experiments on spore dispersal and deposition during the entire cropping period should be conducted as well as climate chamber experiments with spray inoculations after flowering to further investigate whether late occurring spores can infect the plant. The high occurrence of FG and FA in the spore traps might be a result of the similar, slender and curved, spore morphology which might be for aerodynamic reasons more suitable for wind dispersal compared with the globose or napiform spores of FP and FL (Torp and Nirenberg 2004; Leslie and Summerell 2006). Furthermore, we did not observe an effect of the main wind direction comparing grains from border plots with those from plots where infected straw was applied. There is a complete lack of studies about the potential wind dispersal of FP and FL, thus there is a need to clarify whether this is a possible type of transportation.

The reason for the higher occurrence of FL DNA in the oat border plots in 2015 remains unknown. We assume that a higher occurrence of weeds, mainly dicotyledonous, enhanced the colonisation either due to a changed microclimate or as potential alternative hosts, because no herbicide was applied in this year. However, no FL DNA was measured in sampled weeds in the UK, although mainly monocotyledonous plants were sampled (Opoku et al. 2013).

In an *in vitro* study, the rain splash dispersal of FP reached a vertical height of up to 58 cm and a maximum horizontal distance of 70 cm (Hörberg 2002). A dispersal within this distance does not explain the potential cross-contamination between the treatment plots, but could be an explanation for the contamination of the border plots. The spread of FL conidia by rain splash under field conditions remains unknown, but we assume that they behave similar to FP due to the analogue spore morphology (Torp and Nirenberg 2004).

The two oat varieties differed in mycotoxin contamination in the field as Zorro accumulated higher amounts of NIV and T-2/HT-2, Husky accumulated higher amounts of DON. As for other cereal crops, this result indicates that oat varieties also vary in their susceptibility to infection of different *Fusarium* species and accumulation of various mycotoxins.

The average contamination with NIV and T-2/HT-2 in both years was higher compared with the natural contamination of Swiss oats between 2013 and 2015 (refer to chapter 3.4). The higher contamination with DON in the overwintered treatments in 2016 could be a result of a natural infection of the oat crop with FG in 2015. We assume that FG mostly outcompeted FP and FL by faster growth on the residues before and after application in the field, as was also shown in a co-infection experiment (Xu et al. 2007a).

The higher incidence of FP and contamination with NIV in 2016 in the FP and FL field experiments might be a result of lodging due to heavy rainfalls. In fact, in a field study in Japan, a higher NIV concentration was observed in lodged wheat plants artificially inoculated with one FG NIV chemotype and one DON chemotype compared with non-lodged plants (Nakajima et al. 2008). The effect of lodging on FL and T-2/HT-2 contamination in oats is not yet understood and multi-year field studies would be necessary.

The lower contamination with NIV in 2015 and higher contamination with T-2/HT-2 compared with 2016 in the FP and FL field experiments could also be due to different weather conditions. In 2015, a higher precipitation after the straw application, followed by a dry period of six days thereafter, might have been more favourable for the FL development, resulting in more T-2/HT-2 production. At the beginning of anthesis, high precipitation occurred, followed again by dry conditions, which might have also been more favourable for T-2/HT-2 accumulation in 2015, as it was observed in the UK (Xu et al. 2013). However, based on our climate chamber experiment results, the temperatures during anthesis in both years were not favourable for an FL or FP infection.

Still, the observed weather conditions might have been responsible for the higher contamination with NEO in 2015. We assume that NEO was either produced by FL, or that T-2 was metabolised to NEO as observed in several animal species (reviewed in Wu et al. 2010). This kind of metabolism of T-2 to HT-2 was shown in wheat plants, but it is still unknown whether this holds true for oats (Nathanail et al. 2015b). Furthermore, a screening of FL strains on their ability to produce NEO and/or T-2/HT-2 would be valuable to identify potential different chemotypes.

In conclusion, the high contamination with NIV and T-2/HT-2 following incubation under 10 °C treatments and 12 h 99% rH, indicates that epidemiology of FP and FL in oats is markedly different to the well-studied pathogen-host system of FG in wheat. Nevertheless, a similarity to FG and wheat exists in the observation that oat plants at beginning or mid anthesis were more susceptible to FL infection and T-2/HT-2 accumulation compared with growth stages before anthesis. To better understand the environmental requirements and the main dispersal mode of

actions of FP and FL and to better estimate a potential contamination risk in oats, further multi-site field experiments with different oat varieties and various FP and FL strains would be needed as a basis for a forecasting model.

5.6 Acknowledgements

The authors thank Andreas Kägi for outstanding technical support during the field experiments. We thank Eveline Jenny for her support in conducting the molecular work. Excellent support during the sample preparation was provided by Denise Bönisch, Fionna Loeu and Lea Stauber. This work was funded by the Swiss National Science Foundation in the frame of the national research programme 'Healthy Nutrition and Sustainable Food Production' (NRP 69) [grant number 406940_145210]. No potential conflict of interest was reported by the authors.

5.7 Supplementary Material

Table 22: Origin of fungal strains, all isolated in Switzerland from the winter oat variety Wiland.

Fusarium species	Strain number	Isolation year	Geographic origin (community/canton)
F. langsethiae	FL 13005	2013	Merishausen / Schaffhausen
F. langsethiae	FL 13014	2013	Courtételle / Jura
F. langsethiae	FL 14001	2014	Nyon / Waadt
F. poae	FP 13013	2013	St. Urban / Luzern
F. poae	FP 13045	2013	Raperswilen / Thurgau
F. poae	FP 13059	2013	Vers-Chez-Perrin / Waadt

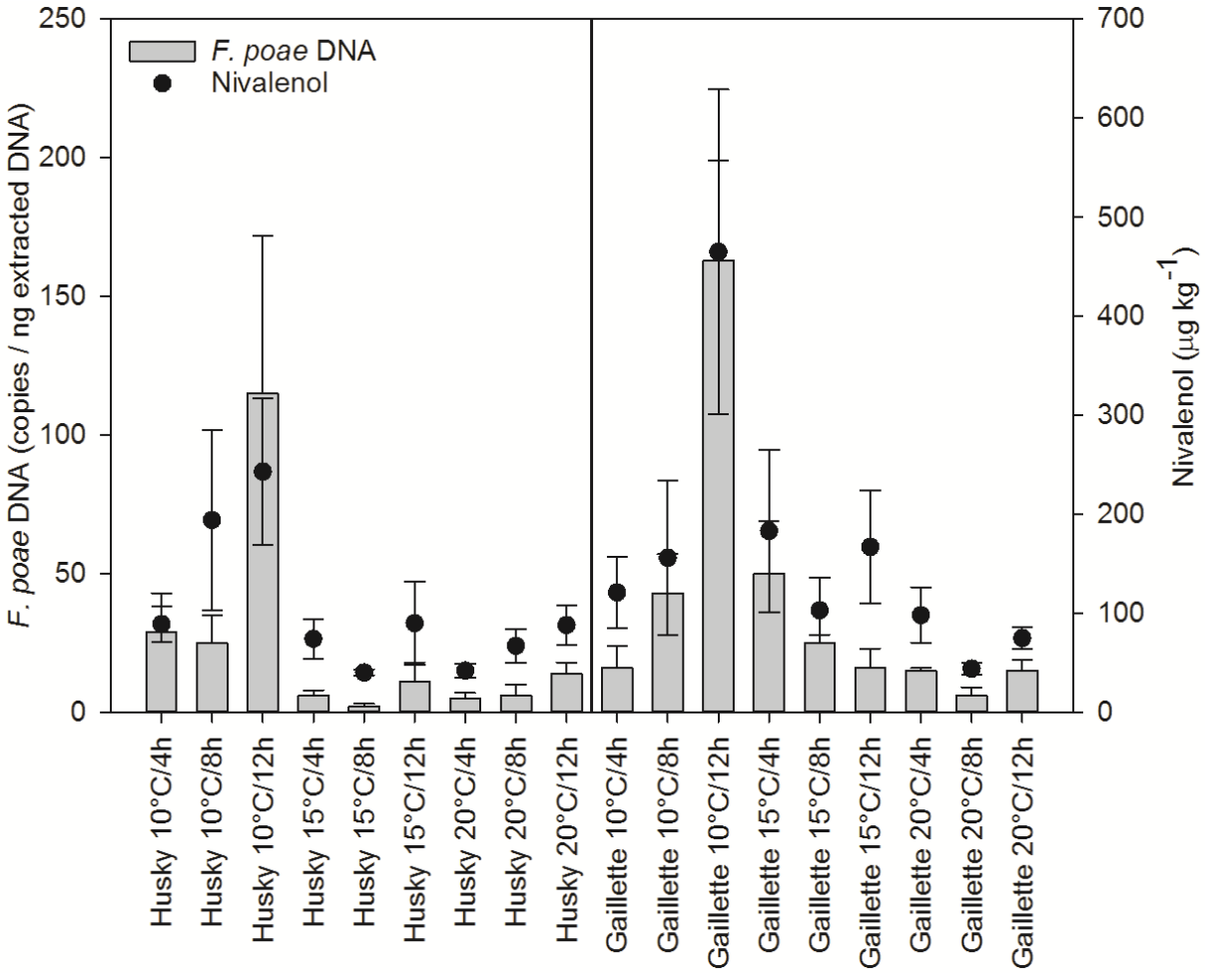

Figure 25: Effect of temperature, duration at 99% relative humidity and variety on *Fusarium poae* (FP) DNA amount (copies/ng extracted DNA) and nivalenol (NIV) content (µg kg^{-1}) in a climate chamber experiment. Pooled data from two experimental runs. n = 81. Analysis were conducted for each variety separately. Error bars represent the standard error of the mean

Table 23: Agilent Mass Spectrometer JetStream Interface parameters.

Mycotoxin	Precursor Ion	Product Ion	Retention time	Fragmentor	Collision Energy	Polarity	Quantifier/Qualifier
NIV	371.0	311.0	2.41	108	4	Negative	Qualifier
NIV	371.0	281.0	2.41	108	8	Negative	Quantifier
DON	355.2	295.2	2.94	95	6	Negative	Quantifier
DON	355.2	59.0	2.94	95	20	Negative	Qualifier
FUS-X	413.1	352.8	3.51	95	8	Negative	Quantifier
FUS-X	413.1	263.0	3.51	65	8	Negative	Qualifier
NEO	400.2	214.8	3.64	70	10	Positive	Qualifier
NEO	400.2	185.0	3.64	70	15	Positive	Quantifier
Ac-DON	356.0	137.0	4.11	95	8	Positive	Quantifier
Ac-DON	339.0	137.0	4.11	105	12	Positive	Qualifier
DAS	384.2	307.1	4.90	75	5	Positive	Quantifier
DAS	384.2	247.0	4.90	105	6	Positive	Qualifier
HT-2	447.2	345.2	5.42	135	14	Positive	Quantifier
HT-2	447.2	285.0	5.42	135	16	Positive	Qualifier
T-2	484.3	215.2	5.74	125	14	Positive	Qualifier
T-2	484.3	185.0	5.74	120	14	Positive	Quantifier
ZEA	371.1	175.0	5.99	190	16	Negative	Quantifier
ZEA	371.1	130.9	5.99	190	24	Negative	Qualifier

NIV: nivalenol, DON: deoxynivalenol, FUS-X: fusarenon X, NEO: neosolaniol, Ac-DON: acetyldeoxynivalenol, DAS: diacetoxyscirpenol, HT-2: HT-2 toxin, T-2: T-2 toxin, ZEA: zearaleone

Table 24: Gradient parameters of the LC-System.

Time [min]	Eluent A [%]	Eluent B [%]
0.00	98.0	2.0
1.00	95.0	5.0
6.50	5.0	95.0
6.75	0.0	100.0
9.50	0.0	100.0
10.00	98.0	2.0
11.50	98.0	2.0

Table 25: Mean relative humidity (%), mean temperature (°C), sum of precipitation (mm) and sunshine duration (h) during different oat growth stages in field experiments of 2015 and 2016.

Year	Time period	DC	Mean relative humidity (%)	Mean temperature (°C)	Sum of precipitation (mm)	Sunshine duration (h)
2015	13.04. – 19.05.	26-51	70	13.5	216	252
2015	20.05. – 05.06.	51-59	70	15.3	17	129
2015	06.06. – 15.06.	61-69	76	18.9	87	64
2015	16.06. – 24.07.	71-92	64	21.2	60	379
2016	11.05. – 01.06.	29-51	77	13.5	152	101
2016	02.06 – 17.06.	51-59	83	15.7	153	58
2016	18.06. – 29.06.	61-69	76	18.8	32	87
2016	30.06. – 04.08	71-92	74	19.7	156	279

* DC 26: Main stem and six tillers; DC 29: Main stem and nine or more tillers; DC 51: Tip of ear just visible; DC 59: Ear emergence complete; DC 61: Beginning of anthesis; DC 69: Anthesis complete; DC 71: Kernel water ripe; DC 92: Grain hard, not dented by thumbnail; according to Zadoks et al (1974).

Table 26: Mean incidence (%) of *Fusarium poae* (FP), *F. langsethiae* (FL) and *F. graminearum* (FG) in oat grains in field experiments from 2015 and 2016, based on seed health tests, described by mean value and standard error (SE) of the mean, averaged over four replicate plots.

Year	Treatment	Variety	FP/FL infection*	% FP ± SE	% FL ± SE	% FG ± SE
2015	Control	Husky	FP	3 ± 1	nd	nd
2015	Freshly inoculated	Husky	FP	3 ± 1	nd	nd
2015	Overwintered	Husky	FP	2 ± 0.4	nd	0.3 ± 0.2
2015	Control	Zorro	FP	8 ± 2	nd	nd
2015	Freshly inoculated	Zorro	FP	7 ± 2	nd	nd
2015	Overwintered	Zorro	FP	6 ± 2	nd	nd
2016	Control	Husky	FP	26 ± 4	nd	4 ± 1
2016	Freshly inoculated	Husky	FP	30 ± 1	nd	2 ± 1
2016	Overwintered	Husky	FP	25 ± 3	nd	8 ± 2
2016	Control	Zorro	FP	42 ± 6	0.3 ± 0.2	5 ± 1
2016	Freshly inoculated	Zorro	FP	33 ± 2	0.3 ± 0.2	6 ± 1
2016	Overwintered	Zorro	FP	32 ± 3	nd	9 ± 2
2015	Control	Husky	FL	4 ± 1	nd	nd
2015	Freshly inoculated	Husky	FL	5 ± 0	nd	nd
2016	Control	Husky	FL	27 ± 5	nd	5 ± 2
2016	Freshly inoculated	Husky	FL	22 ± 3	nd	5 ± 1
2016	Overwintered	Husky	FL	16 ± 2	nd	11 ± 4
2016	Control	Zorro	FL	29 ± 4	nd	11 ± 2
2016	Freshly inoculated	Zorro	FL	29 ± 4	2 ± 1	7 ± 2
2016	Overwintered	Zorro	FL	25 ± 2	nd	17 ± 2

* FP/FL infected straw applied in the field; nd: not detected

6 General discussion

Fusarium species belong to the most important cereal pathogens, not only because of the broad host range and the wide geographical distribution, but mainly due to the production of health threatening mycotoxins. Numerous studies were conducted on *Fusarium* species in wheat, but comparatively little has been investigated in other cereals. Because cereal species differ in their *Fusarium* species spectrum, the present study was conducted to investigate the dominant species and main produced mycotoxins in barley and oats. Furthermore, influencing cropping factors that affect the infection were examined. These overall objectives were approached by monitoring studies from farm samples and subsequent analysis.

A second objective was to elucidate epidemiological factors, mainly temperature and humidity, which affect the infection of barley and oats with the most occurring or main toxin producing *Fusarium* species. The following discussion will highlight and critically review the achieved results and applied methods.

6.1 Occurrence of *Fusarium* species and mycotoxins in Swiss barley and oats

The most prominent *Fusarium* species in Swiss wheat is *F. graminearum* (FG) (Vogelgsang et al. 2011), whereas the species complex of Swiss barley and oats was unknown. Monitoring studies have been conducted to examine the main occurring *Fusarium* species and cropping factors that influence their occurrence.

We observed that the species complex in barley comprises up to nine different species, with FG being the most dominant, as observed in Swiss wheat (Vogelgsang et al. 2009). The second and third most detected species were *F. avenaceum* and *F. poae* (FP), respectively. This species spectrum was also observed in other European countries, such as the UK, Germany or France, although FG was found to be less dominant in these studies (Nielsen et al. 2014; Ioos et al. 2004; Linkmeyer et al. 2016). This might be a result of different climatic conditions as reviewed in Osborne and Stein (2007) and/or to different collection approaches and monitoring years.

Furthermore, Linkmeyer et al. (2016) and Nielsen et al. (2014) used molecular methods to distinguish the different *Fusarium* species in barley, while we used the seed health test (SHT) method. The advantage of this method is that the whole *Fusarium* spectrum can be determined without cost intensive methods such as multiplex quantitative PCR (qPCR). Certainly, one

disadvantage of the SHT method is that slow growing species, such as *F. langsethiae* (FL) (Torp and Nirenberg 2004) or FP, might be overgrown by faster growing species such as FG, as shown in a co-inoculation experiment (Xu et al. 2007b). In addition, the flour used for qPCR might contain a more diverse spectrum compared with the 100 grains used in the SHT. Hence, the occurrence of other *Fusarium* species might have been underestimated, even though in-house test showed a good correlation between qPCR and SHT results for FP, FL and FG.

Deoxynivalenol (DON) was the predominant mycotoxin in the barley samples, which is mainly produced by FG (Desjardins 2006) and indeed correlated well with the FG incidence. The dominance of DON was also observed by Linkmeyer et al. (2016) in Germany and by Nathanail et al. (2015a) in Finland. Despite its dominance, the DON mean concentrations during the monitoring years 2013 and 2014 were low because the overall warm and dry weather conditions were not favourable for a FG infection. The maximum limit of 1'250 µg kg^{-1} for unprocessed cereals (The European Commission 2006) was only exceeded in 10 out of 440 samples. However, stronger contaminations in barley have been reported (Hietaniemi et al. 2016; Beccari et al. 2017) and might also occur in Switzerland.

In Swiss oats, the overall species spectrum was different than that of barley. Here, the main occurring *Fusarium* species was FP, whilst the second most occurring species differed between the three monitoring years. In 2013 it was FG, in 2014 *F. avenaceum* and in 2015 FL. In fact, FP was also frequently isolated from oats in other European countries, such as UK, Sweden and Norway (Edwards et al. 2012; Fredlund et al. 2013; Hofgaard et al. 2016a).

In addition to the SHT data, we conducted quantitative PCR (qPCR) for FP and FL, because T-2/HT-2 were the predominant mycotoxins in oats followed by nivalenol (NIV), while only a low occurrence of FL was observed. This is in line with other oat studies, reporting high values of these toxins (Edwards et al. 2009; Hofgaard et al. 2016a). The strong correlation of FL DNA with T-2/HT-2 in the monitoring samples, confirmed FL as an important producer in Europe as was also reported in other countries (Fredlund et al. 2013; Hofgaard et al. 2016a). The other known producer of T-2/HT-2 is *F. sporotrichioides* which occurs only occasionally in Europe (Bernhoft et al. 2010; Edwards et al. 2012; Kosiak et al. 2003). However, the European Commission indicative level for unprocessed oats with husk of 1'000 µg kg^{-1} (The European Commission 2013) was only exceeded in 17 out of 325 samples. Due to the reduced knowledge about FL, it is not known whether a higher contamination could occur as a result of particular weather conditions. Remarkably, the typical FHB symptom is not observed in oats which makes a visual assessment impossible. Thus, healthy looking grains might contain elevated levels of mycotoxins (Imathiu et al. 2013a; Stenglein 2009).

The reason for the low occurrence of NIV with the high occurrence of FP in the oat samples remains unknown. Probably NIV is not a virulence factor for FP as was claimed by some authors with respect to DON for FG (Ilgen et al. 2008; Bai et al. 2002). The higher T-2/HT-2 contamination might be explained by the assumption, that FL is a strong toxin producer. Another hypothesis is that it was disturbed by other species, which triggered a higher toxin production. The potential role of T-2 or HT-2 as a virulence factor for FL is not determined yet. In summary, it was shown that barley and oats differed with respect to their mainly occurring *Fusarium* species. As of today, it is not fully understood why different *Fusarium* species have a preference either for barley or for oats. However, it is assumed that the different morphology of the florescence of the respective cereal species is responsible for this phenomenon (Opoku et al. 2013).

6.2 Influence of the main cropping factors on *Fusarium* species incidence and mycotoxin contents in barley and oats

Agronomic factors such as crop rotation, tillage, choice of variety and fungicide application have been shown to successfully control FHB caused by FG (Blandino et al. 2012). In addition, a combination of different control measures showed synergistic effects and there is consensus, that an integrated management is the best strategy for a sustainable control (Wegulo et al. 2015). The analysis of the barley samples revealed a strong effect of the previous crop maize on the FG incidence and DON content. The infection of maize by FG and the saprophytic survival on maize residues which can serve as an inoculum for the following crop are well known for wheat (Osborne and Stein 2007; Dill-Macky and Jones 2000) and confirm our results for barley. A further increase of FG incidence and DON was observed after a cultivation of maize two years in a row, which probably resulted in an even higher disease pressure.

The decaying of plant material by microorganisms is favoured by ploughing, due to a closer contact in the soil (Pereyra and Dill-Macky 2008) and in turn might reduce the fungal inoculum. This was particularly noticed in this study in combination with the previous crop maize, although only a reduced FG incidence but not a reduced DON content was observed. The reason for this result could be that either the remaining infecting FG strains were potent DON producers, or that the differences were too small, because of the generally low average incidences and toxin contents in both years. Besides the negative consequences of reduced or

no tillage on FHB, many positive effects are known for soil quality and fertility, such as increase of organic matter, less erosion and promotion of soil organisms (Uri et al. 1998; Dill-Macky 2008).

A further inoculum reducing measure was the chopping of crop residues on both, the FG incidence and the DON content, as observed in a multi-year study with wheat following grain maize (Vogelgsang et al. 2011). The reduced particle size most probably accelerated the decomposition, although this effect was not observed in a study conducted with maize leaves (Vestergaard et al. 2001). Other hypotheses are that earthworms can access the chopped residues more easily and thus reduce the above ground inoculum (Wolfarth et al. 2011). Moreover, a stimulated growth of other competing fungi due to the enlarged surface could have reduced the colonisation of the crop residues with FG.

In Switzerland, barley is mainly grown in the Extenso system, which prohibits the usage of fungicides. Furthermore, even in conventional Swiss cropping systems, an application of fungicides in barley is only allowed before anthesis. We observed, that this pre-anthesis application even enhanced the FG infection, since other fungi were probably eliminated and thus FG did not have to compete with them for space and nutrients.

The main influencing factor for a T-2/HT-2 contamination in oats was the previous crop small grain cereals, which was also observed in Norway and in the UK (Bernhoft et al. 2012; Edwards et al. 2009). Even though only low levels of FL DNA were detected in cereal residues in a field experiment in Norway (Hofgaard et al. 2016b), it is assumed that FL can survive on cereal straw.

Tillage was found to be a further influencing factor for the T-2/HT-2 content. Samples from ploughed soils showed lower contamination compared with samples from reduced tillage fields. If the above assumption will be approved by future studies, the results might be due to a reduction of the inoculum source.

Overall, the influencing factors for FP and NIV were not as clear as for FL and T-2/HT-2, and reduced NIV contents in samples from ploughed fields were observed only in one monitoring year. Nevertheless, a lower content of both mycotoxins was detected in spring sown compared with autumn sown oat samples. This was also observed in a monitoring study in the UK and in a field experiment in Lithuania (Suproniene et al. 2010; Edwards et al. 2009). This finding might be explained by a potential longer growing period, which also prolonged the colonisation time for the *Fusarium* species. Another reason might be that the spring sown oat varieties are less susceptible to infection compared with the autumn sown varieties. Still, the cultivation of spring oats is often accompanied by a reduced quality and yield and is thus not appealing for

farmers. These inoculum and infection reducing control measures are even more important for oat growers, since an application of fungicides against *Fusarium* species in Swiss oats is not permitted.

Both monitoring datasets need to be interpreted with caution, since the samples were obtained from several fields with a different agronomic history. In addition, the samples were taken by the respective farmers, who might not always have followed the provided instructions on how to take a representative subsample. Nonetheless, these data are a valuable starting point for future well-defined field experiments to investigate the effect of selected agronomic factors.

6.3 Influence of climatic conditions on the infection of *Fusarium graminearum* in barley and of *F. poae* and *F. langsethiae* in oats

Knowledge about the most influencing weather conditions that affect an infection are essential to predict mycotoxin contaminations in grains and thus minimise potential food and feed safety hazards. Hence, climate chamber experiments were conducted to investigate the influence of combinations of particular temperatures and humidity durations at 99% rH on FG infection in spring barley and FP and FL in spring oats.

In barley, incubations at 15 °C led to the highest FG severity and DON contamination. This finding is in contrast to studies with wheat, where temperatures above or equal to 20 °C were shown to result in higher infections (Osborne and Stein 2007; Xu et al. 2007a), but might be due to the earlier anthesis of barley compared with wheat, which is usually accompanied by lower temperatures. The FG strains that infect barley might be more adapted to these cooler conditions, to achieve an optimal infection.

The prolonged incubation at 99% rH (8 and 12 h) did result in increased FG severity, FG DNA amount and DON content under all tested temperatures for the variety Concerto. However, for variety Ascona, this increase was only observed at 20 °C. The longer humidity duration probably enhanced the time for germination and thus the time frame to infect the plants, as has been also observed in other studies (Xu et al. 2007a; Martínez et al. 2012; Beyer et al. 2005).

The climate chamber experiment with barley exposed several issues. The detection of anthesis is not as easy as compared with wheat, since barley is flowering while it is still in the boot stage. Hence, after appearance of the first anthers, the inoculation was made, even though the ear was not fully emerged. In some plants, the inoculation was thus probably made at DC 69 instead of

DC 65 (Zadoks et al. 1974), which might have influenced the infection. Also, the data from the first repetition of the experiment could not be used, since the inoculated ears were mouldy and not suitable for further analysis. Hence, in repetition two and three, ears were gently removed from the flag leaves two days after inoculation, if they were still partly covered.

For oats, an incubation at 10 °C was the most favourable for an infection with FP and FL. In accordance with our results, less coleoptile growth of FP infected wheat was observed at 10 °C and 15 °C compared with 20 °C and 25 °C, which implies an enhanced infection rate of FP under cooler conditions (Brennan et al. 2003). Still, a direct comparison of these results with the current study is difficult, because two different disease patterns were evaluated. Furthermore, in other studies, a higher FP and FL infection was observed with rising temperatures (Xu et al. 2007a; Opoku et al. 2013), which might be due to the facts that Xu et al. (2007a) investigated FP in wheat and that Opoku et al. (2013) investigated FL under field conditions, where other factors are expected to have a greater impact on disease development.

In plants inoculated at 10 °C, a higher FP DNA amount and NIV contamination with prolonged duration of 8 h and 12 h 99% rH was observed, as it was also found in wheat (Xu et al. 2007a). The prolonged wet period might have expanded the period of conidia germination and infection.

The impact of humidity duration on the infection with FL is not well understood. A successful inoculation was obtained by bagging oat shoots for six days after spray inoculation (Divon et al. 2012), although such a long period of high humidity is not realistic under field conditions. In contrast to our results with FP, the current experiments with FL demonstrated that the infection was already successful after 4 h 99% rH and only slight increases were observed with a prolonged humidity duration.

Until recently, it was not known, whether the main infection period for FL is during or before oat anthesis. In an experiment under controlled conditions, only a low amount of FL DNA was found after inoculation during the booting stage before anthesis (Divon et al. 2012). In commercially produced oats, more FL biomass was detected during head emergence but before flowering (Opoku et al. 2013), however in contrast to our study plant material harvested at head emergence was analysed and not grains after harvest. Our study implies, that the main FL infection is during anthesis, however, we did not test whether an FL infection is also possible after anthesis. This might hold true because Hofgaard et al. (2016b) detected more FL DNA in spore traps at time points of four weeks after heading.

Since we only tested one oat variety the data needs to be interpreted with caution. It is possible that oat varieties differ in their susceptibility to FL as it was shown for FG (Tekauz et al. 2004),

and in the UK, a higher susceptibility towards FL infection was observed in winter oats compared with spring oats (Edwards 2009).

6.4 Spore deposition and dispersal of *Fusarium graminearum*, *F. poae* and *F. langsethiae*

The observed spore dispersal before anthesis revealed a higher occurrence of FG spores compared with the spore dispersal during anthesis. It is suggested, that spores that are washed into the flag leaf bearing the flowering ear are also sheltered and the moist conditions inside might enhance the infection (McCallum and Tekauz 2002; Osborne and Stein 2007). Hence, the higher spore dispersal before anthesis might have caused the infection. Unfortunately, data on the spore deposition are only available for 2016, due to sampling errors in 2015.

In a one-year field experiment where barley plants were artificially spray inoculated with a FG spore suspension at different growth stages, it was shown that the most susceptible stages were DC 51, 56 and 65. In contrast, in a greenhouse experiment by Yoshida et al. (2005), cleistogamous barley varieties were shown to be more susceptible after anthesis. This finding suggests a potentially longer susceptible period of barley compared with wheat (Wegulo 2012).

No difference for the DON content was observed between the years, which was an unexpected result, since a higher FG severity and FG incidence was noticed in 2016 compared with 2015. The higher precipitation after anthesis until harvest in 2016 might have resulted in a leaching of the water soluble DON (Kushiro 2008), as has been observed in studies in Austria, the USA and Switzerland (Lemmens et al. 2004a; Gautam and Dill-Macky 2012; Schenzel et al. 2012).

In oats, in both years, very few deposited spores of FP or of FL were detected. The most detected species were FG and *F. avenaceum* which might have overgrown the slow growing species FP and FL, which was shown for the former in a co-inoculation experiment (Xu et al. 2007b).

In the field experiments with inoculated FP and FL straw, no difference to the control treatment was observed. This suggests, that spores were either transported within the field or entered from outside the field. The observation of potential wind dispersal or influencing weather factors for FP and FL is also more difficult compared with FG, because it is not known whether these species produce ascospores and whether these are somehow ejected. A contamination by spores coming from outside is possible, but the knowledge about alternative hosts is sparse. Until to date, FL was isolated from weeds (Opoku et al. 2013) and FP from various plant species apart

from cereals, such as tomato and gramineous weeds (Landschoot et al. 2011; Stenglein et al. 2009) but the main inoculum source as well as the overwintering strategy remain unclear.

As of today, it is not clear whether FP could be transported by splash dispersal and in fact, an *in vitro* study showed that FP spores can reach a maximum height and horizontal distance of 58 and 70 cm, respectively (Hörberg 2002). Studies about the splash dispersal of FL are missing, but it is assumed that they might behave similar to FP, due to the similar spore morphology (Torp and Nirenberg 2004).

The spore trap method bears some challenges that should be addressed: A heavy occurrence of deposited spores renders it difficult to distinguish and to count colonies and thus needs to be approximated based on the counts of two eights of a given plate. Also, slugs frequently entered the traps, feeding on the agar, probably removed or transported spores and contaminated the plates with excrements. Hence, the employment of a spore trap using Eppendorf Tubes® as described in Hofgaard et al. (2016b), and a subsequently conducted qPCR could overcome these issues.

7 Conclusions

The present study revealed FG and DON as the main *Fusarium* species and mycotoxin in Swiss barley, whereas in Swiss oats, FP and T-2/HT-2 were the most dominant *Fusarium* species and mycotoxins. Since the combination of factors frequently resulted in an increased infection with the respective *Fusarium* species, the entire cropping system should be taken into account to reduce the infection risk. For barley, the cultivation of maize in the previous year should be avoided and if this rotation needs to be used, ploughing or additional chopping of maize residues should be conducted. In parallel, for oats, small grain cereals should not be grown as a previous crop and ploughing should be performed. If possible, sowing of spring varieties is preferable because in barley and oats, the winter varieties revealed a higher *Fusarium* incidence and mycotoxin content compared with spring varieties.

In addition to the impact of cropping factors, a strong influence of temperatures but no clear effect of humidity durations on the *Fusarium* species infections in barley and oats was demonstrated. The latter finding implies, that an inoculation for 4 h at 99% relative humidity is sufficient for an infection, which might represent a high risk of subsequent contamination, even under conditions that are usually not favourable for fungal development. The study on FL inoculations during different growth stages of oats revealed the beginning and mid of flowering as the most susceptible growth stages. Due to insufficient data, the spore deposition of FG in barley fields is not yet fully understood. The inoculum sources of FP and FL remain unknown, as no colonies were detected during anthesis and no differences were detected between the treatment and the border plots. Thus, the present dataset is insufficient to develop a forecasting system for FP and FL in oats and requires further studies, whereas a simple model for FG in barley could be tested and improved within the next years.

8 Outlook

The obtained dataset from the current monitoring of Swiss barley and oats provided a highly valuable insight in the species spectrum and allowed to elucidate the most important influencing agronomic factors. However, a continued monitoring should be conducted to examine whether a potential shift in the *Fusarium* species complex arises and to determine potential site specific differences within Switzerland. The effect of the already identified influencing factors should be investigated in multi-year and multi-site field experiments to evaluate the results under standardised situations. Furthermore, the best risk-reducing crop rotations should be examined towards their applicability under commercial farming conditions. Studies about the differences between winter and spring varieties of barley and oats are needed to identify the reasons for the reduced contamination in the spring varieties. This could also include the effect of phenological parameters such as open versus closed flowering, time of anthesis as well as plant height and time of booting. These parameters could contribute important data to be used in future barley and oat breeding programs. Since some oat varieties seemed to be more contaminated with DON than with NIV or T-2/HT-2, experiments on the susceptibility of different oat varieties towards different *Fusarium* species and toxin accumulation are of crucial importance. To better understand the interactions between different FHB causing species and to explain the phenomenon of high T-2/HT-2 or NIV contaminations together with low DON contaminations and vice versa, thorough co-inoculation studies should be conducted to possibly identify potential niches that are used by particular species. In addition, to clarify the reason for the observed correlation between neosolaniol contamination and FL DNA, it would be important to examine the possible existence of different FL chemotypes and to establish a FL database, as has been done for European FG strains (Pasquali et al. 2016).

Alternative spore trap methods as those used by Hofgaard et al. (2016b), could help to obtain more knowledge about the inoculum source and spore deposition of FG in barley as well as of FL and FP in oats. To understand the wind and/or rain splash dispersal of FL and FP, experiments that allow to trace the inoculum with microsatellites and to collect spores at different heights within the oat field, would be of utmost importance. Furthermore, to identify the overwintering strategies and primary infection sources of FL and FP, the study of potential alternative hosts, including weeds or grass borders among the fields would be required. This study would also include the analyses of soil and root samples on their presence of FL and FP DNA, to exclude the possibility of soil born inoculum, as a result of the saprophytic survival

on roots of the previous crop. In addition, climate chamber experiments with different barley and oat varieties inoculated before, during and after anthesis at different growth stages with spore suspensions of FG and FP/FL, respectively, should be conducted to verify whether a potential longer susceptible growth period exists and whether late infections can occur. Based on both, weather conditions and influencing agronomic factors, existing forecasting systems should be further developed and extended to sustainably reduce the risk of contaminated oat and barley grains, and thus to ensure food and feed safety.

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V Acknowledgements

During the previous years of my PhD studies, I got invaluable support by various people who I would like to thank at this point.

First of all, I am particularly thankful to my supervisor **Dr. Susanne Vogelgsang**, for her kind support, enthusiasm and encouragement in guiding me through this project and correcting uncounted numbers of pages, posters and presentations without losing her patience.

I would also like to thank **Prof. Dr. Beat Keller** for leading my dissertation at the University of Zurich and **Prof. Dr. Christoph Ringli** for being member of my PhD committee.

I am also particularly thankful to the Healthy and Safe consortium: **Tomke Musa, Dr. Fabio Mascher, Dr. Thomas D. Bucheli and Mario Bertossa** for their constructive comments and critical feedback on my work. In addition, I would like to thank **Charlotte Martin**, for her support during the whole project and the time we spent not talking about science.

Moreover, I would like to thank **Evelyne Jenny** for her support during the molecular analysis, **Irene Bänziger** for introducing me to the morphological identification of *Fusarium* species, **Dr. Hans-Rudolf Forrer** for sharing his broad and valuable experience and his critical comments on my work, **Andreas Kägi** for his always helping hands, **Dr. Felix E. Wettstein** for his instructions to the LC-MS/MS methods and **Philipp Streckeisen** for his assistance during my greenhouse and climate chamber experiments. Furthermore, the **Agroscope Reckenholz field group** for their support during my various field experiments.

I would also like to acknowledge all trainees, "Zivis" and master students who supported me during my work: **Denise Bönisch, Fionna Loeu, Joyce Meissner, Karin Kibler, Lea Stauber, Georg Oberer and Fabian Hess**.

A special thanks goes to the whole "**Ökis**" Group with their former and present members (Dr. Susanne Vogelgsang, Tomke Musa, Evelyne Jenny, Irene Bänziger, Deborah Kaiser, Lara Reinbacher, Dimitrios Drakopoulos, Andreas Kägi, Alejandro Gimeno, Pascal Weber, Dr. Giselher Grabenweger, Dr. Hans-Rudolf Forrer and Christian Schweizer). Without your support in scientific matters and especially time beyond the research with amusing coffee breaks, apéros and excursions, it would have felt like real work.

My warmest thanks are addressed to my **family** for their unconditional support and for always believing in me.

The last and biggest thanks is addressed to my wife **Anita**. Not only for helping me with the layout of the thesis but mainly for supporting and taking care of me.

I thank **YOU**, if I forgot to mention you.

VI Curriculum vitae

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Martin, C.; **Schöneberg, T**.; Vogelgsang, S.; Vincenti, J.; Bertossa, M.; Mauch-Mani, B.; Mascher, F. (2017) Factors of wheat grain resistance to Fusarium head blight, *Phytopathology Mediterranean*, 56, 154-166

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